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Investigating Present-day Health Issues of the American Lobster (*Homarus americanus*)

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INVESTIGATING PRESENT-DAY HEALTH ISSUES OF THE AMERICAN LOBSTER

(HOMARUS AMERICANUS)

By

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B.S. University of Maine, 1983

A DISSERTATION

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Doctor of Philosophy

(in Aquaculture and Aquatic Resources)

The Graduate School

The University of Maine

August 2018

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(*HOMARUS AMERICANUS*)

By Deborah Anita Bouchard

Dissertation Advisor: Dr. Robert Byer

An Abstract of the Dissertation Presented
in Partial Fulfillment of the Requirements for the
Degree of Doctor of Philosophy
(in Aquaculture and Aquatic Resources)
August 2018

The American lobster, *Homarus americanus*, H. Milne Edwards, 1837, supports the most economically valuable fishery along the North Atlantic coast of North America. A collapse in lobster populations in Southern New England (SNE) has coincided with increasing ocean temperatures and emerging diseases. This research investigated the etiologies of limp lobster disease (LLD) and epizootic shell disease (ESD), two diseases that continue to cause significant mortality in natural lobster populations. Mortality from LLD is associated with the bacteria *Photobacterium indicum* and is more intense in impounded lobsters. To more clearly define the community ecology of this suspected opportunistic pathogen, the microbial biofilms of freshly captured and impounded adult lobsters from the Northern Gulf of Maine coastal shelf were surveyed and compared. *P. indicum* was found to be a common member of the microbial communities of freshly captured and impounded *H. americanus* and was also isolated from the lobster pound sediment. There is no medicated feed that is approved for use in controlling *P. indicum*. Therefore, a *P. indicum* bacterin was produced and used as an immunostimulant to stimulate a short-term protective immune response in adult American lobsters. The

prepared bacterin was safe to use with lobsters and no adverse reactions were observed. Epizootic shell disease (ESD) is an aggressive form of shell disease likely involving multiple microbes. A laboratory study was performed to examine the effects of three seasonal temperature cycles on the immune response and progression of ESD in adult female American lobsters. There was a trend of increased mortality in the diseased lobsters in the mid- and high-temperature seasonal cycles. Both temperature and shell disease influence bacterial loads. The culture dependent microbial diversity remained relatively stable over time and temperature suggesting that increasing temperatures alone did not exacerbate ESD progression or alter microbial communities. Taken together, this work increases our understanding of the etiology of the factors that could influence population abundance and disease emergence in lobsters.

DEDICATION

To my mentors who inspired and believed in me:

Susan Sergeant, Dr. Daphne Stoner,

Dr. Darrell Pratt, and Dr. Paul Reno

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CHAPTER 1

INTRODUCTION

Overview of the American Lobster Fishery

The American lobster, *Homarus americanus*, H. Milne Edwards, 1837, is an iconic marine decapod crustacean that represents one of the most important invertebrates ecologically, culturally, and economically on the northeastern coast of North America. *H. americanus* is an important ecological contributor to subtidal community dynamics as predator and prey. Culturally, the lobster fishery represents a way of life for generations of families and coastal communities in the Northeast. In 2016, lobster landings in the United States exceeded 158 million pounds with an estimated value of 666.7 million dollars, representing one of the most valuable commercial fisheries on the Atlantic coast (2018). However, U.S. lobster stocks have been on diverging trajectories. While current stock abundance in the Gulf of Maine and Georges Bank remain high, Southern New England (SNE) stocks have declined steeply over the last ten years and are described as having collapsed and being in recruitment failure (ASMFC, 2015). Today, the Maine lobster fishery is considered to be a well-regulated and sustainable resource. However, it is unrealistic to believe that this will continue indefinitely as the lobster fishery is vulnerable to the same environmental and ecological fluctuations that drive the abundance and distribution of all species (Holland, 2011). Infectious disease agents exist in the marine environment and environmental changes have been linked to frequency of disease outbreaks (Burge et al., 2014). In SNE, population declines have coincided with trends of increasing temperatures and emerging diseases. In the context of climate

change, it is critical to investigate the impacts of a changing environment and the prevalence and impact of diseases on this extremely important lobster fishery.

History of Diseases in American Lobster

Compared to many other decapod crustacean species, *H. americanus* has relatively few known infectious disease agents (as reviewed by (Cawthorn, 2011, Behringer et al., 2012, Shields, 2012). Historically, some of the most documented diseases of the American lobster include gaffkemia, bumper car disease, impoundment shell disease and burnt spot shell disease. In brief review, the causative agent of gaffkemia is the bacterium, *Aerococcus viridans var homeri* (Vachon et al., 1981, Bayer and Daniel, 1987), and bumper car disease is caused by the ciliate, *Anophroides haemophila* (Greenwood et al., 2005). With impoundment shell disease and burnt spot shell disease, the causative agent or agents are thought to be bacterial or fungal in nature but the exact etiology remains undefined (Smolowitz et al., 2005). Gaffkemia, bumper car disease and impoundment and burnt spot shell disease are primarily diseases associated with impounded lobsters and are most likely the result of microbial agents that gain an advantage to produce disease through anthropogenic stressors such as handling, trauma, impoundment high densities, water quality, and temperature changes (Cawthorn, 2011).

Over the last two decades, new and emerging infectious diseases and disease syndromes in lobsters have coincided with recognized environmental stressors such as pollution, poor water quality and temperature extremes (Shields, 2012, Shields, 2013). These emergent diseases have caused significant mortality in natural populations. Those

with measurable economic impact include neoparamoebiasis, limp lobster syndrome, and epizootic shell disease (ESD). All present with complex etiologies and all are described as possible indicators of climate change (Cawthorn, 2011, Behringer et al., 2012, Shields, 2012). The marine amoeba, *Neoparamoeba pemaquidensis*, is the agent associated with neoparamoebiasis in lobsters that occurred during a Western Long Island Sound (WLIS) mortality event in 1999 (Mullen et al., 2004). This amoeba is ubiquitous in marine environments and can be found on healthy lobsters. However, it is considered the proximate cause of the high mortality in WLIS which was also experiencing many extreme environmental stressors probably compromising the health of the lobster and creating an opportunity for the amoeba to be infectious (Mullen et al., 2004, Cawthorn, 2011, Shields, 2013). Limp lobster syndrome, given this name because caught lobsters display weakness and lethargy, was first described in the Gulf of Maine during the late 1990s and early 2000s, as a highly invasive bacterial disease that was 'not' gaffkemia (Tall et al., 2003). Although the bacterial agent thought to cause the now termed 'limp lobster disease'(LLD) was first identified as *Vibrio fulvialis* (Tall et al., 2003), later work revealed the causative bacterial agent to be *Photobacterium indicum* (Basti et al., 2010b). Mortalities from the disease still occur today in natural populations but more intensely in impounded lobsters (Francis *pers com*). The question remains whether *P. indicum* is an emerging pathogen or an opportunistic pathogen of highly stressed lobsters (Basti et al., 2010b, Cawthorn, 2011). ESD emerged in the later part of 1996 in SNE and by 2000 was considered an epizootic that spread along the northeastern coast. ESD is described as a 'severe erosive shell disease affecting the

dorsal carapace of *H. americanus* (Smolowitz et al., 2005). Disease characteristics suggest a bacterial etiology but the causative agent or agents have not been elucidated (Castro et al., 2012, Chistoserdov et al., 2012, Meres et al., 2012, Quinn et al., 2013). The prevalence of ESD remains at medium to high levels in SNE (Castro and Somers, 2012) and has appeared with greater frequency in New England waters mainly south of Cape Cod. Since 2011 Maine has seen a slight rise in the increase of ESD but overall prevalence levels remain at less than 2% compared to SNE rates of greater than 30% (<http://www.maine.gov/dmr/science-research/species/lobster/2016monitoring.html>).

Research Objectives

The purpose of these studies is to gain additional insight into the etiologies of LLD and ESD, two American lobster diseases that continue to cause significant mortality in natural populations. In order to more clearly define the community ecology of *Photobacterium indicum*, the suspected pathogen in LLD, a survey of the culturable microorganisms in the biofilms of freshly captured and impounded *H. americanus* from the Northern Gulf of Maine was performed. As an alternative to antibiotic treatment for mitigating lobster mortality in pounds due to *P. indicum* and other possible gram negative pathogens, a *P. indicum* bacterin was evaluated as an immunostimulant to stimulate the innate and cellular response of adult American lobsters. With ESD, a repeated measures study was performed in order to better define the influence of temperature on the immune functions and the progression of ESD in lobsters. Along with contributing new insight into the etiologies of these two diseases, outcomes of this research may provide an alternative for control for bacterial infections in impounded

lobsters and provide a more thorough understanding of the consequences of elevated ocean temperatures.

CHAPTER 2

CULTURE-DEPENDENT SURVEY OF THE MICROBIOOTA OF THE ADULT LOBSTER (*HOMARUS AMERICANUS*)

Introduction

The American lobster *Homarus americanus* (H. Milne Edwards, 1837) supports one of the most valuable commercial fisheries in the Northwest Atlantic. In the State of Maine, the 2015 live landings of 55,539 metric tons represents the highest in recorded history (Maine State Department of Marine Resources, 2017). The continued abundance of harvestable lobsters throughout most of the Gulf of Maine, despite intense fishing pressure, and the projections for the recruitment of juveniles into the population, suggests that the fishery is sustainable into the near future (ASMFC, 2015). This is in part due to effective management strategies, and the decline of ground fish predators such as cod fish (*Gadus morhua* L.), and other gadoids.

Lobsters are captured in baited traps, which are recovered and marketable lobsters are then moved to market. In the late summer and fall, lobster pound owners purchase lobsters from fishermen and over-winter them in pounds to be released as the market demand increases. A lobster pound is a three-sided embayment that is part of the natural shoreline, with the fourth side being a gate to the open ocean, allowing for semidiurnal flushing (tidal water exchange) (Figure 2.1). Pound owners often maintain a record of transactions that can extend as far back as 50 years or more. The loss or mortality of lobsters, colloquially termed “shrinkage”, is determined by the difference in total weight of lobsters at full stocking density minus the weight of lobsters that are

harvested. The application of this method over the years by pound owners has resulted in it becoming a fairly reliable indicator of changes in inventory and therefore lobster survivability. Pound losses appear to be increasing over the last 20 years despite no clear evidence of a disease epizootic, and in the face of widespread antibiotic use. Medicated feed in the form of oxytetracycline dihydrate is approved by the FDA to be used prophylactically to control *Aerococcus viridans var homari*, the causative agent of gaffkemia (“red tail” disease) historically reported as a major cause of mortality in impounded lobsters during the 1970’s and 1980’s (Vachon et al., 1981, Bayer and Daniel, 1987). Despite continued active and passive disease surveillance, *A. viridans* has not been detected in the Maine post-capture lobster population since 2006, or in pounds and land-based holding facilities in Atlantic Canada during this time (Bouchard et al., 2010, Basti et al., 2010b). However, a gram negative bacterium, *Photobacterium indicum* (Xie and Yokota, 2004), has been isolated from the hemolymph in pure culture at a 3%-5% prevalence of approximately 6,000 individuals, and in greater than 95% of morbid and dead lobsters over this 5 year period. This isolate may represent an emerging opportunistic pathogen of stressed lobsters destined for long distance transport or storage in Maine tidal impoundments (Basti et al., 2010b).



Figure 2.1. Lobster pound

The objectives of this study are to survey and compare the culturable microorganisms in the biofilms of freshly captured and impounded adult lobsters from the northern Gulf of Maine coastal shelf, and to more clearly define the community ecology of the suspected opportunistic bacterial pathogen *P. indicum*.

Materials and Methods

Lobster Sampling and Sediment Collection

A total of 120 adult, market-size lobsters were collected through fall and winter from three impoundment sites in southern, midcoast and northern Maine. At each site, 40 lobsters were sampled consisting of an arbitrary subset of 20 individuals from freshly captured lobsters that were held overnight at dockside in submerged crates and 20 resident lobsters that were dredged from the pound. Impounded lobsters had a minimum residence time of 30 days.

Lobster Sampling: Samples for culture-dependent analysis were taken from two anatomical sites on each lobster. The following procedure applied to all sampled lobsters which consisted of sampling the branchial chamber, essentially the gills, and the distal intestinal tract. Each live animal was gently held ventral side up, and a sterile 14 cm polyester tipped applicator (Puritan Medical Products Co. LLC, Guilford, ME. 04443 USA) was oriented perpendicular to the long axis of the body and carefully directed to the gill lamellae. This method allowed for the sampling of the medial aspect of the carapace by proximity, and the gill lamellae. A second new sterile applicator was carefully directed into the anus, rectum and distal intestine to a depth of approximately 5-7 cm. All applicators were placed into 0.9 mls of a sterile phosphate buffered saline solution (Lonza, Walkersville, MD. USA), held on ice and transferred back to the laboratory.

Sediment Sampling: Sediment sampling was performed by using a sterile 50 ml conical tube, sinking it into the sediment of the pound at the edge of the water at low tide and scooping up a full tube of sediment.

Sample Processing

Lobster samples: Each applicator in sterile phosphate buffered saline solution was vortexed for 10 seconds. The applicator was discarded and a sterile 10 μ l inoculating loop (Simport, Beloeil, Qc J3G-4S5, Canada) was used to streak a plate of Trypticase Soy Agar with 5% sheep red blood cells +1.5% NaCl (BA) (Northeast Laboratory, Waterville, ME. USA), and a Marine Sea Salt Agar plate (MSA), prepared in house with 10.0 g Trypticase Soy Agar (Becton, Dickinson and Co. Sparks, MD. USA),

20.0g agar (Becton, Dickinson and Co. Sparks, MD. USA), 300 ml Instant Ocean®, Aquarium Systems), 700 ml deionized water, and then autoclaved for sterility. While this bacterial sampling was not intended as an enumeration method, the size of the loop, hence the inoculum, indicated that observed growth was in numbers higher than 10^3 colony forming units per milliliter. All plated samples were incubated aerobically at 16° C for 48-72 hours.

Sediment samples: Ten grams of sediment were diluted in 90 ml of sterile phosphate buffered saline, vortexed, and 10 µl of the solution was transferred using a sterile 10 µl inoculating loop onto the 2 non-selective media. All plated samples were incubated aerobically at 16° C for 48-72 hours.

Bacterial identification: At the end of the incubation period, predominant isolates were selected from each media source and anatomic site based on similar colony morphology, media reaction (hemolysis, agarolysis) and pigment production. Isolated colonies were re-streaked onto the corresponding medium for re-isolation and purity and the isolates incubated again for 24-48 hrs at 16°C. Preliminary biochemical profiling included Gram stain, vibriostatic 0129 susceptibility (150 µg 2,4-diamino-6,7-di-isopropylpteridine phosphate, Oxoid, Hampshire, England) oxidase, catalase reaction and carbohydrate utilization and H₂S production with triple sugar iron (TSI) medium. Isolates were then filtered according to preliminary similar biochemical reactions prior to final identification using the Biolog® Microbial Identification System Gen II (BMIS). The BMIS uses a 96 well plate format to test 95 discrete substrates. Patterns of utilization are compared to an established data base of characteristic reaction patterns

for identification purposes (www.biolog.com). Isolates categorized as CDC group II-E subgroups A and B and CDC group II-H by BMIS were sent out for 16S ribosomal DNA sequence analysis and identification.

Results

In this study, microbial sampling resulted in over 1300 bacterial isolates being identified using standard bacterial phenotypic profiling (gram stain, oxidase, O129 vibriostat and carbohydrate utilization). These results allowed similar organisms to be grouped, and subsets further identified to the genus and species level using the Biolog Microbial Identification System (BMIS). We were able to culture and phenotypically identify 77 species affiliated with 36 genera, 23 families, 7 classes/orders and 5 phyla. The Biolog Microbial Identification System provides identification by means of phenotypic/biochemical characteristics. BMIS results range in probability of identification and can provide high probability to the species level while some results provide a genus with moderate to low probability of species and others to genus only.

Using the described media, the predominant culturable and identified bacteria in the intestinal mucosa and branchial chambers of adult lobsters captured or impounded in the Northern Gulf of Maine coastal waters are *Beta* and *Gamma Proteobacteria* representing 46 of the 77 total species identified. Table 2.1. presents the species, genera, families, class and phyla identified and represented. Approximately 12% of the 1300 cultured bacteria could not be identified by BMIS to the genus level as there were too few reactions to obtain an identification. An overview of genera identified in the

intestinal mucosa, the branchial chamber (gills) and the pond sediment for each medium type is presented in Figure 2.1

Table 2.1. Lobster bacterial isolates.

Phylum	Class	Family/Order	Genus	Species
Actinobacteria	Actinomycetales	Brevidibacteriaceae	Brevibacterium	Brevibacterium mcbrellineri
		Corynebacteriaceae	Corynebacterium	Corynebacterium nitrilphilus Corynebacterium urealytica
	Actinobacteria	Intrasporangiaceae	Kytococcus	Kytococcus sedentarius
		Microbacteriaceae	Arthrobacter	Arthrobacter cumminsii Arthrobacter histidinovorans
			Kocuria	Kocuria rosea
			Micrococcus	Micrococcus luteus
			Curtobacterium	Curtobacterium citreum
			Dermacoccus	Dermacoccus nishinomiyaensis
		Tsukamurellaceae	Tsukamurella	Tsukamurella inchoensis
Bacteroidetes	Flavobacteriia	Flavobacteriaceae	Chrysobacterium	Chryseobacterium indoltheticum Chryseobacterium scophthalmum
			Empedobacter	Empedobacter brevis
			Flavobacterium	Flavobacterium ferrugineum Flavobacterium flevense Flavobacterium mizutaii
				Flavobacterium xanthum
				Flavobacterium xanthum
	Sphingobacteria	Sphingobacteriaceae	Sphingobacterium	Sphingobacterium spiritovorum Sphingobacterium thalophilum
			Reimerella	Reimerella anatipestifer
Deinococcus- Thermus	Deinococci	Deinococcaceae	Deinococcus	Deinococcus radiodurans Deinococcus radiophilus

Table 2.1. Continued

Phylum	Class	Family/Order	Genus	Species
Firmicutes	<i>Coccus</i>	<i>Saphylococcaceae</i>	<i>Staphylococcus</i>	<i>Staphylococcus capitis</i> <i>Staphylococcus xylosus</i>
	<i>Bacilli</i>	<i>Bacillaceae</i>	<i>Bacillus</i>	<i>Bacillus sphaericus</i>
Proteobacteria	<i>Alphaproteobacteria</i>	<i>Brucellaceae</i>	<i>Ochrobactrum</i>	<i>Ochrobactrum anthropi</i>
		<i>Sphingomonadaceae</i>	<i>Sphingomonas</i>	<i>Sphingomonas adhaesiva</i> <i>Sphingomonas parapaucimobilis</i> <i>Sphingomonas paucimobilis</i> <i>Sphingomonas sanguinis</i>
				<i>Delftia acidovorans</i>
				<i>Aquaspirillum dispar</i> <i>Aquaspirillum metamorphum</i> <i>Aquaspirillum peregrinum ss integrum</i>
	<i>Gamaproteobacteria</i>	<i>Neisseriaceae</i>	<i>Delftia</i>	
			<i>Aquaspirillum</i>	
			<i>Chromobacterium</i>	<i>Chromobacterium violaceum</i>
			<i>Iodobacter</i>	<i>Iodobacter fluviatilis</i>
		<i>Aeromonadaceae</i>	<i>Aeromonas</i>	<i>Aeromonas hydrophila</i> DNA group 1 <i>Aeromonas hydrophila</i> DNA group 2 <i>Aeromonas schubertii</i> DNA group 12, <i>Aeromonas veronii/sobria</i> DNA group 8
		<i>Moraxellaceae</i>	<i>Psychrobacter</i>	<i>Psychrobacter immobilis</i>
		<i>Pasteurellaceae</i>	<i>Pasteurella</i>	<i>Pasteurella anatis</i> <i>Pasteurella bettyae</i>

Table 2.1. Continued

Phylum	Class	Family/Order	Genus	Species
Proteobacteria	Gamaproteobacteria	Pseudoalteromonadaceae	Pseudoaltermonas	<i>Pasteurella volantium</i>
				<i>Pseudoalteromonas</i>
				<i>nigrifaciens</i>
				<i>Pseudomonas alcaligenes</i>
				<i>Pseudomonas bathycetes</i>
				<i>Pseudomonas fluorescens</i>
				<i>biotype G</i>
				<i>Pseudomonas savaastonoii</i> pv
				<i>fraxini</i>
				<i>Pseudomonas syringae</i> pv <i>pisi</i>
		Shewanellaceae	Shewanella	<i>Pseudomonas tolaasii</i>
				<i>Shewanella algae</i>
				<i>Shewanella putrefaciens</i>
		Vibrionaceae	Listonella	<i>Listonella anguillarum</i>
				<i>Listonella pelagia</i>
				<i>Photobacterium damsela</i> ss
			Photobacterium	<i>damsela</i>
				<i>Photobacterium indicum</i>
			Vibrio	<i>Vibrio aestuarianus</i>
				<i>Vibrio alginolyticus</i>
				<i>Vibrio carcariae</i>
				<i>Vibrio furnissii</i>
				<i>Vibrio harveyi</i>
				<i>Vibrio metschnikovii</i>
				<i>Vibrio parahaemolyticus</i>
				<i>Vibrio proteolyticus</i>
				<i>Vibrio splendidus</i>
				<i>Vibrio tubiashii</i>
				<i>Vibrio vulnificus</i>

For the intestinal mucosa, species from 21 of the 36 genera were identified. *Vibrio* species are the predominant genus isolated. The next six most prevalent genera in descending frequency of isolation are *Pasteruella*, *Photobacterium*, *Burkholderia*, *Aeromonas*, *Acidovorax* and *Chrysobacterium* species. While there is a significant number of unidentified isolates in the intestinal mucosa they cannot be grouped into specific genera. The branchial chamber has a more diverse assemblage of genera and species with 35 of the 36 genera represented. While *Vibrio* species are predominant, they are closely followed in descending frequency of isolation by *Pseudomonas*, *Acidovorax*, *Burkholderia*, CDC group, *Aeromonas* and *Sphingomonas* species. Again, there is a significant number of unidentified isolates in the gills that cannot be grouped to specific genera. The three sediment samples tested had the least diverse assemblage with 6 of the 36 genera represented. The genus isolated and identified were *Photobacterium*, *Vibrio*, *Arthrobacter*, *Pseudomonas*, *Sphingobacterium* and *Tsukmurella* species with similar frequency of isolation. The isolates categorized as CDC group II-E subgroups A and B, and CDC group II-H and identified by 16S ribosomal DNA sequence analysis were predominantly *Pseudoaltermonas* and *Flavobacterium* species. Not all CDC group isolates were able to be identified by 16S ribosomal DNA sequence analysis. These results are accounted for in the presented results in Table 2.2 for frequency of isolation.

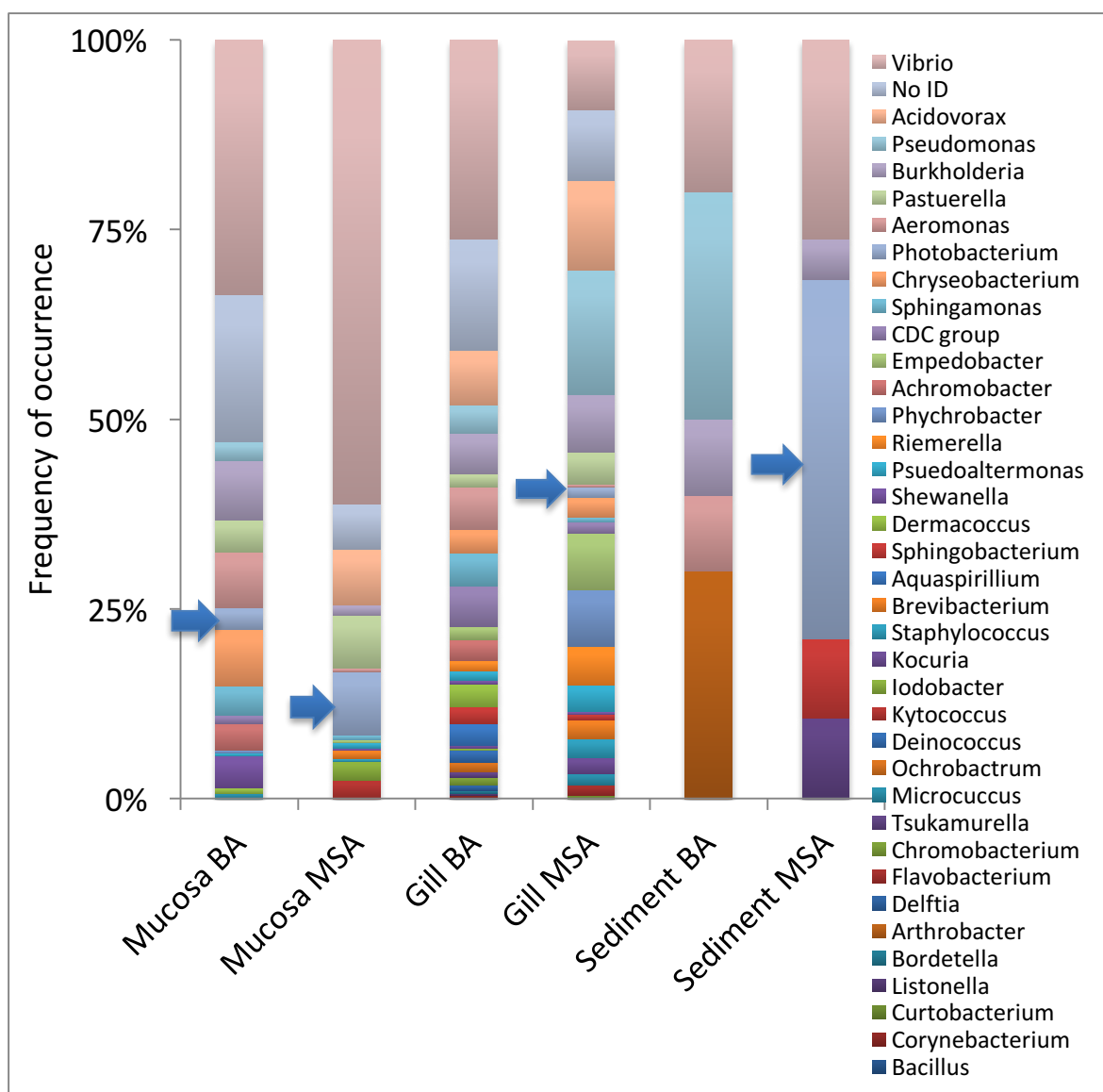


Figure 2.2. Frequency of bacterial isolates to genus on selected media. Trypticase Soy Agar with 5%sheep red blood cells +1.5% NaCl (BA), Marine Sea Salt (MSA). Arrows indicate *Photobacterium* species.

Discussion

This project utilized traditional non-selective media and phenotypic/biochemical characteristics to culture and identify bacteria within the biofilms of *Homarus americanus*. Culture-independent metagenomic analyses using 16S rDNA gene profiles have revolutionized the identification of unculturable microbes, and have revealed that

conventional techniques, may vastly under-represent the true abundance and diversity of microbes within biofilms. In fact, the more easily cultured bacteria may not be the most abundant (Muyzer et al., 1993, Hiergeist et al., 2015). However, our main objective was to survey and compare the culturable microorganisms in the biofilms of adult lobsters from the northern Gulf of Maine coastal shelf, and to more clearly define the community ecology of the suspected opportunistic bacterial pathogen *Photobacterium indicum*.

In defining culturable, the media selected plays a large role in the microorganism's ability to grow. This study utilized two different media, Trypticase Soy Agar with 5% sheep red blood cells +1.5% NaCl (BA) and Marine Sea Salt Agar (MSA). The BA medium is considered to be for more fastidious organisms while the MSA is a low nutrient medium that allows for the growth of a broader range of marine organisms. Both media select for aerobic and facultative anaerobic organisms. Our observations suggest that the most commonly cultured bacteria isolated from the intestinal mucosa of adult *Homarus americanus* are *Proteobacteria* in the family *Vibrionaceae*. The gill lamellae and the medial aspect of the cephalothoracic carapace are also dominated by *Proteobacteria*, with a more diverse assemblage represented by the *Flavobacterium-Cytophaga-Bacteroides*, *Firmicutes* and *Actinobacteria* divisions. A similar pattern of dominance and diversity is evident in freshly captured lobsters and in long-term impounded residents. Although sampling was performed over a five-month period, it is difficult to draw inferences about changes in microbial community structure based on different water temperatures, habitats and geography. These findings are in

agreement with other researchers working with *Nephrops norvegicus* (Meziti et al., 2010), *Panulirus argus* (Porter et al., 2001), *Penaeus merguensis* (Oxley et al., 2002) and other Penaeid species (Dempsey et al., 1989). Sediment samples also reflect the ubiquitous distribution of genera within the *Proteobacteria* division. Many of these cultured bacteria are considered human and animal opportunistic pathogens, although no coliforms were isolated. This was also noted in a survey of naturally occurring bacteremia in Long Island Sound lobsters (Bartlett et al., 2008).

Photobacterium indicum is a gram-negative, facultatively aerobic, pleomorphic rod in the family *Vibrionaceae*. It is 0129 vibriostat susceptible, oxidase-positive and reacts as alkaline/acid on triple-sugar iron agar. It appears to be a common member of the microbial communities of the branchial chamber and intestinal mucosa of freshly captured and impounded *H. americanus*, and has also been isolated from the sediment adjacent to lobster pounds. However, it is unknown if it is a transient colonizer or a permanent commensal resident of the lobster. An *in vitro* temperature dependent growth profile (Bouchard et al, unpublished data) indicates that the growth of this organism slows dramatically below 12° C and above 23° C. This may be a partial explanation for the “seasonality” of mortalities observed by pound-owners, with a peak occurring in the late fall as water temperatures in the pound are decreasing (Basti, 2008). A *Vibrio fluvialis*-like bacterium that was implicated as the cause of an outbreak of ‘limp lobster disease’ in Maine during 1997-1998 (Tall et al., 2003) was subsequently identified by (Giray and Bouchard, 2002), as *Hyphomicrobium indicum*. *H. indicum* has been more recently transferred to the *Photobacterium* genus as *Photobacterium*

indicum (Xie and Yokota, 2004). *Photobacterium* species are widely distributed in the marine environment, and have been isolated from seawater, sediment and the tissues of a variety of marine animals. A comprehensive review of the phylogeny, genomics and symbiotic relationships of the genus *Photobacterium* may be found in (Urbanczyk et al., 2011).

Aerococcus viridans, the causative agent of the disease Gaffkemia, is a gram-positive cocci that was initially described in 1947 (Snieszko and Taylor, 1947) and is considered the most significant opportunistic pathogen of wild and impounded lobsters in the northern hemisphere. The most recent estimates of the overall mean prevalence of *A. viridans* in the hemolymph of lobsters sampled in Atlantic Canada are 6.30% with a 95% confidence interval of 3.64 - 10.03% (Lavallee et al., 2001). It is interesting to note that *A. viridans* has not been isolated in this study, or reported as the cause of a major disease outbreak in a holding facility in the Northwest Atlantic for at least a decade. A culture-dependent survey of healthy and shell diseased lesions in *H. americanus* conducted in southern New England and Long Island Sound, also failed to isolate *A. viridans* (Chistoserdov et al., 2005). (Bouchard et al., 2010) proposes that infectious pressure by *A. viridans* may have driven the evolution of *Homarus americanus* by selecting for disease resistance, or the bacterium may be in an avirulent phase of a natural cycle. These observations underpin the fact that host-microbe interactions are not always predictable (Casadevall et al., 2011).

In conclusion, the information obtained in this culture-dependent survey is relevant in the context of obtaining and characterizing the more easily cultured bacteria

from the biofilms of adult lobsters. Performing 16S ribosomal DNA sequence analysis on all isolates unable to be identified by the BMIS may increase the number of specific genera and species profile but available databases for all identification systems also play a critical role in identification confirmation (Wragg et al., 2014). We would also recommend that, in light of this work, the prophylactic use of oxytetracycline dihydrate in lobster impoundments is contraindicated in the absence of a clear diagnosis of mortality caused by *Aerococcus viridans*

CHAPTER 3

THE IMMUNE RESPONSE OF THE AMERICAN LOBSTER (*HOMARUS AMERICANUS*) TO CHALLENGE WITH A *PHOTOBACTERIUM INDICUM* BACTERIN

Introduction

Traditionally, the use of antibiotics has been an important strategy to control bacterial diseases in finfish and crustacean aquaculture when applied in a rational manner consistent with physical findings, laboratory culture, and antibiotic susceptibility. Lobsters live within an aqueous milieu of microorganisms, resulting in constant exposure to possible pathogenic and opportunistic infections through direct contact or ingestion. The American lobster industry has access to an FDA approved medicated feed in the form of oxytetracycline dihydrate to be used prophylactically to control *Aerococcus viridans var homari*, the causative agent of gaffkemia (“red tail” disease). Gaffkemia has been historically reported as a major cause of mortality of impounded lobsters (Vachon et al., 1981, Bayer and Daniel, 1987). Despite the widespread use of the approved medicated feed, recent documented reports have indicated an increase in mortality of lobsters during impoundment not associated with gaffkemia (Basti, 2008, Tall et al., 2003). *Photobacterium indicum* (Xie and Yokota, 2004) has been consistently isolated from moribund and dead lobsters in pound surveys and may present an emerging opportunistic pathogen of stressed lobsters destined for long distance transport or storage in Maine tidal impoundments (Basti et al., 2010b). The results from Chapter 2 of a culture dependent survey of the microbiota of the adult American lobster indicated that *P. indicum* was a common member of the microflora of

the adult lobster as well as the pond environment, further supporting the opportunistic nature of this organism.

The enactment of stricter regulations regarding antibiotic usage in Europe, North America and Japan is in recognition of the spread of multiple antibiotic-resistance determinants between bacteria due to excessive, inappropriate and prophylactic antibiotic use, and of the threat to human health (Defoirdt et al., 2011). Using antibiotics prophylactically to control aquatic animal disease is not sustainable and can be ineffective. Novel alternatives to control bacterial infection are therefore a major area for further research in disease control in aquaculture (Subasinghe et al., 1998, Smith et al., 2003, Ayisi et al., 2017). Recent literature is replete with information regarding probiotics, prebiotics and synbiotics being developed and used in aquaculture to mitigate disease and increase protection (Verschuere et al., 2000, Smith et al., 2003, Rowley and Pope, 2012, Ayisi et al., 2017). In crustaceans, alternatives to antibiotics have focused on the use of glucans, lipopolysaccharides and live, killed and/or components of bacterial cells as immunostimulants (Smith et al., 2003, Sang and Fotedar, 2010).

It has long been considered that crustaceans lack an adaptive immune system. Like other decapod crustaceans, the disease defense mechanisms of *H. americanus* rely on an innate, or non-specific immune response, which has cellular and humoral components (Bowden, 2017). Paterson and Stewart were two of the first researchers to examine the cellular responses of *H. americanus* hemocytes using *A. viridans* and *Pseudomonas perolens* bacterins in order to stimulate an immune response (Paterson

and Stewart, 1974, Paterson et al., 1976, Paterson and Stewart, 1979). Results from their work suggested that exposure of lobsters to these bacterins increased the phagocytic activity of lobster hemocytes with a higher response being observed with the gram negative *P. perolens* and a *P. perolens* endotoxin (Paterson et al., 1976). Further research was performed using bacterins to induce protection against gaffkemia in impounded and natural populations (Keith et al., 1992, Stewart et al., 2004). While protection was realized, it was only for a relatively brief duration of immunity (less than 93 days), indicating an innate immune response was responsible for protection. However, offering protection by stimulating a protective immune response for even a short time period could be beneficial (Hauton, 2012).

This study seeks to determine the efficacy of a *P. indicum* bacterin as an immunostimulant to stimulate the innate and cellular response of adult American lobsters. It is hypothesized that *H. americanus* will exhibit a measurable immune response when exposed by immersion to the *P. indicum* bacterin, and that this response may provide short-term protection to vulnerable lobsters challenged with *P. indicum* or other gram-negative opportunistic pathogens.

Materials and Methods

Preparation of Bacterin

Photobacterium indicum, isolated from an infected Maine lobster and DNA sequenced by Micro Technologies Inc. Richmond, Maine, was streaked for isolation on Trypticase Soy Agar with 5% Sheep's Blood + 1.5% NaCl (BA) and then allowed to incubate at 16°C to match optimal growth characteristics of *P. indicum* (Bouchard et al

unpublished data) for 24 hours. Isolated colonies were inoculated into two 18 mm glass tubes containing 20 mls of sterile Trypticase Soy Broth + 1.5% NaCl. The tubes were gently shaken at 16°C for 24 hours and then aseptically transferred into 1.0 L of sterile TSB +1.5% NaCl and shaken at 16°C for 24 hours. The 1 L of inoculated broth was then scaled up to a final volume of 18.8 L with sterile TSB +1.5% NaCl and shaken and aerated at 16°C for 48 hours. Serial dilutions and plate counts determined that there were 10^9 CFU ml⁻¹ of bacteria in the mixture. The bacterial culture was then inactivated by adding 5.0 ml of 37% formalin L⁻¹ to the bacterin mixture, and allowing it to sit at room temperature for 48 hours. A viability test was then performed to ensure that there were no live bacteria by adding 100 µL of the inactivated mixture to 10.0 mls of sterile TSB + 1.5% NaCl and incubating at 16°C for 5 days and monitoring for bacterial growth.

Lobster Husbandry

Lobsters were individually compartmentalized to reduce agonistic behavior and cannibalism within a 5400-liter artificial seawater (Crystal Sea® Marinemix, Baltimore, MD) recirculating system. Water was mechanically and chemically filtered and passed through a bank of four 65 W ultraviolet sterilizers. Water temperature was maintained at 15°-16°C and the light/dark duration kept at the ambient light cycle. Water quality was monitored by spectrophotometry (Hach Company, Loveland, CO) and was maintained according to the following recommended levels with partial water changes and foam fractionation. Total ammonia nitrogen and unionized ammonia less than 1.0 mg/L and 0.3 mg/L respectively at a pH of 7.8–8.1; nitrite, less than 0.1 mg/L; salinity, 33–35 ppt; S.G., 1.023–1.025; and oxygen, > 7.0 mg/L. All lobsters were individually fed

every 48 hours on an alternating schedule of cooked Maine shrimp and locally caught frozen smelt.

Safety Testing of the *Photobacterium indicum* Bacterin

Safety testing was performed to determine if adult *H. americanus* would demonstrate any adverse reactions to immersion exposure to the formalin inactivated cells and lipopolysaccharide of *P. indicum*. The experiment was conducted on lobsters acclimated for 14 days to artificial seawater at 15°C. Forty lobsters (mean weight 450–550 g) from a group of 49 were randomly allocated into treatment groups consisting of 10 lobsters each as presented in Table 3.1. The remaining nine lobsters served as non-treatment controls.

Table 3.1. Safety testing treatments. Trypticase Soy Broth (TSB), Artificial Sea Water (ASW), not applicable (n/a).

Treatment #	Composition	Volume (L)	Volume ASW (L)	Dilution
1	Bacterin + TSB	6	24	1:5
2	Bacterin + TSB	3	27	1:10
3	TSB only	6	24	1:5
4	Control	0	30	n/a

Five lobsters in each treatment group were immersed for 5 minutes and the remaining five in each group were immersed for 10 minutes. All lobsters were rinsed in fresh artificial sea water prior to being returned to the holding system. Lobsters were monitored three times a day for weakness or mortality for 7 days. All weak lobsters and mortalities were sampled for bacteriology by aseptically collecting a 100-μL aliquot of

hemolymph from the dorsal abdominal artery and plating the hemolymph onto BA and incubating at 16°C for 48 hrs.

Experimental Design of *Photobacterium indicum* Bacterin Immersion

Freshly captured market-size lobsters (n = 150; mean weight, 450–550 g) were obtained from a lobster pound. Twenty lobsters were randomly sampled for post capture “stressed” hematology immediately upon arrival at the experimental holding facility. All lobsters were then allowed to acclimate at 16° C for 2 weeks. At the end of the acclimation period, 33 lobsters were sampled for “acclimated” hematology for time zero. Following a 48-hour recovery period from blood draw, lobsters were randomly assigned to one of four treatment groups (identified with color-coded numbered tags) consisting of 35 lobsters (Table 3.2). Lobsters were dipped in the following treatment solution for 60 minutes. Treatment group 1 (TSB + bacterin) consisted of *P. indicum* bacterin formalin inactivated in the TSB + 1.5% NaCl. Treatment 2 (sea water washed bacterin) formalin inactivated *P. indicum* cells in TSB + 1.5% NaCl that were centrifuged and washed three times in 35 ppt sterile artificial seawater (Crystal Sea® Marinemix, Baltimore, MD) and re-suspended in sterile artificial seawater. Treatment 3 (sea water immersion control) 35 ppt sterile artificial seawater (Crystal Sea® Marinemix, Baltimore, MD). To prepare the dips for each treatment, 6L of the treatment solutions were mixed with 24 L of artificial seawater (Crystal Sea® Marinemix, Baltimore, MD) to make a total of 30L. Then lobsters were dipped in each treatment for a total of 60 minutes with aeration. In addition, Treatment 4 (no immersion control) was used that consisted of

lobsters being housed in the same manner as the experimental lobsters without exposure to the dipping procedure.

Table 3.2. Bacterin treatments. Trypticase Soy Broth (TSB), Artificial Sea Water (ASW), not applicable (n/a).

Treatment #	Composition	Volume (L)	Volume ASW (L)	Dilution	Immersion Time
1	Bacterin + TSB	6	24	1:5	60 min
2	Washed Bacterin + seawater	6	24	1:5	60 min
3	Seawater control	0	30	n/a	60 min
4	Control no immersion	n/a	n/a	n/a	n/a

All lobsters were sampled at 12, 36, 60, 108 (4.5 days) and 252 (10.5 days) hours post-immersion. At each time point, all lobsters from all treatment groups were sampled to obtain hemolymph for hemocyte population differentiation and total hemocyte counts. Fifteen lobsters from each treatment group were randomly sampled for live cells obtained from hemolymph for the respiratory burst assay.

Hematology Sampling

The optimal anticoagulant and handling procedures to maintain cell viability were selected for this project based on the results of extensive in-house experimental trials with several anticoagulants reported in the literature (Smith and Soderhall, 1983, De Guise et al., 2005, Moss and Allam, 2006). The final choice of anticoagulant for *H. americanus* was the crustacean anticoagulant (CAC) described in Moss & Allam 2006. For all hematology sampling, approximately 1.5 milliliters of hemolymph were collected aseptically from the dorsal abdominal artery into a sterile 3.0 cc syringe with a 23-gauge needle, and then discharged into a chilled glass tube (Figure 3.1).



Figure 3.1. Hemolymph sampling

A 200 μ l aliquot of hemolymph was then reallocated into a chilled 1.5 ml centrifuge tube containing 800 μ L of 10% neutral buffered formalin. Neutral buffered formalin was prepared from 10 ml of 37% (w/v) stock formaldehyde diluted in 90 ml of filtered 35 ppt artificial seawater. Care was taken to prevent contact of the hemolymph with the sides of the vial, which could cause hemocyte agglutination or lysis. Vials were stored at 4°C until analyzed. An additional 400 μ l of hemolymph was collected aseptically from the ventral abdominal sinus in syringes preloaded with 1.6 ml crustacean anticoagulant (CAC) to be analyzed for respiratory burst on the fluorescent plate reader.

Total Hemocyte Counts (Fixed Cells) by Fluorescence-Activated Cell Sorting (FACS)

FACS is a specialized form of flow cytometry that allows for high throughput sampling of cells. In this experiment, a method to measure the total number of hemocytes and to differentiate hemocyte populations was established using flow

cytometry, formalin fixed hemocytes and counting beads. This was adapted from a method by Inoue *et al.* (2002), for teleost leukocytes. For absolute cell counting, CytoCount™ 5.2µM polystyrene fluorospheres (DakoCytomation) were added to the formalin fixed hemocyte suspension at a final concentration of 100 particles µl⁻¹ sample in a total volume of 2ml just prior to flow cytometry analysis. Cytocount™ fluorospheres were gated on a forward scatter (FSC) versus side scatter (SSC) scattergram and all events counted and recorded until 5000 fluorospheres had been acquired. For hemocyte differentiation, total hemocytes were distinguished and gated on an FL-1 versus SSC scattergram by their FL-1 intensity. These hemocytes were then plotted on an FSC versus SSC scattergram in order to differentiate between hemocyte populations. The absolute cell count of hemocytes µl⁻¹ of hemolymph was calculated using the following equation:

Number cells µl⁻¹ hemolymph = (no. of events per population) x 100^A / 5000^B x 100^C Where A is the concentration of fluorospheres added to sample and B is the number of fluorospheres counted and C the dilution factor of hemolymph sample.

In order to cross-check hemocyte counts obtained by flow cytometry, a subsample of the formalin fixed hemocytes (n = 104) was used to correlate absolute hemocyte counts made by flow cytometry and those from microscopy counts. Total hemocyte counts were performed with a Kova Glasstic slide 10 with quantitative grids (Hycor Biomedical Inc., Garden Grove, CA). Pearson's correlation was used to determine the correlation between the outcome results of hemocyte counts by microscopy and flow cytometry.

Respiratory Burst Assay

Respiratory burst activity was measured in live hemocytes *in vitro* by a fluorometric 96 well plate assay adapted from Moss and Allam (2006), that utilizes a non-fluorescent dye (di-hydrorhodamine) that becomes oxidized by hydrogen peroxide (H_2O_2) to a form a detectable fluorescent dye called rhodamine during the cell's respiratory burst. Solutions were prepared according to ((Smith and Soderhall, 1983), where 40mg zymosan was added to 2mL sterile seawater for a final concentration of 20 mg/mL, boiled for 30 minutes, washed by centrifugation twice at 700 xg for five minutes and re-suspended in 2mL sterile marine crustacean saline (MCS). Zymosan concentrations were counted in triplicate using a hemocytometer and stored at 4 °C until use. Dichlorofluorescein di-acetate (DCFH-DA, Sigma) dissolved in DMSO to a obtain 100 mM stock solution was aliquoted and stored at -20 °C. Working solutions of DCFH-DA were produced by diluting aliquots in filter-sterilized (0.22 μ m) autoclaved seawater.

Approximately 400 μ L of hemolymph was collected aseptically from the ventral abdominal sinus in syringes preloaded with 1.6 ml crustacean anti-coagulant CAC (CAC; 0.45M NaCl, 0.1M glucose, 0.3M trisodium citrate, 0.026M citric acid, and 0.01M EDTA, pH 4.6). Samples were centrifuged for five minutes at 200 xg at 5°C and re-suspended in 1 mL sterile marine crustacean saline (MCS; 0.58M NaCl, 0.013M KCl, 0.013M $CaCl_2$, 0.026M $MgCl_2$, 0.00054M N_2HPO_4 , and 0.05M Tris-HCL buffer, pH 7.6). Using a hemocytometer to determine cell counts, cell numbers were adjusted to 1×10^6 cells/mL. Six wells per lobster were plated in a 96 well plate with 100 μ L at 15 °C in the

dark. DCFH-DA was added at a final concentration of 0.5 mM to each well. Plates were read using a BioTek plate reader via fluorescence at 485nm excitation and 535nm emission. Finally, prepared zymosan stock was added to triplicate wells at a ratio of 50:1 zymosan:hemocytes. MCS was added to triplicate wells as a control for unstimulated activity of the hemocytes. Plates were incubated at 15 °C in the dark and read as previously described at 5, 30, 60, and 90 minute intervals after the addition of zymosan. Respiratory burst was calculated as a difference in fluorescence between readings.

Statistical Analyses

Hemocyte counts of stressed and acclimated lobsters were compared using a Mann-Whitney test. For each lobster the percentage change in hemocyte counts and optical densities (respiratory burst assay) was calculated, where baseline values were considered 100%, by dividing the hemocyte counts or optical densities from lobsters ten days post dip by the counts or optical densities after acclimation prior to the dipping treatments then multiplied by 100. The percentage change for hemocyte counts and optical densities were then compared across the four treatment groups with ANOVAs. Analyses were conducted using SPSS v. 24.0.

Results

Safety Testing of the Observation *Photobacterium indicum* Bacterin

The observation period for the safety study was 7 days. During this time, a total of 3 mortalities were observed as follows: 1 control lobster (5 minute seawater only immersion), 1 lobster from the Bacterin + TSA treatment group (1:5 dilution, 10 minute immersion) and 1 lobster from the TSA only treatment group (1:5 dilution, 10 minute

immersion). All mortalities were abacteremic in culture and there were no specific abnormalities noted at post-mortem. These results suggest that the putative bacterin and its constituents are safe in lobsters by immersion and ingestion at both dilutions and exposure times.

***Photobacterium indicum* Bacterin Challenge Trial**

The duration of the *P. indicum* bacterin trial was 252 hours (10.5 days). A total of 4 mortalities were observed as follows: 3 lobsters from the Bacterin + TSB group (2 during molting), and 1 lobster from the control group (no immersion). All mortalities were abacteremic in culture and there were no specific abnormalities noted at post-mortem. This again suggests the safety of the putative bacterin.

Fluorescence-Activated Cell Sorting to Determine Total Circulating Hemocyte Counts μl^{-1} Hemolymph

The differentiation of hemocyte populations of *H. americanus*, via flow cytometry, was not possible. The contour plots for hemocytes based on size and complexity (granularity) demonstrate that the hemocyte populations are not distinguishable by FACS (Figures 3.2 and 3.3).

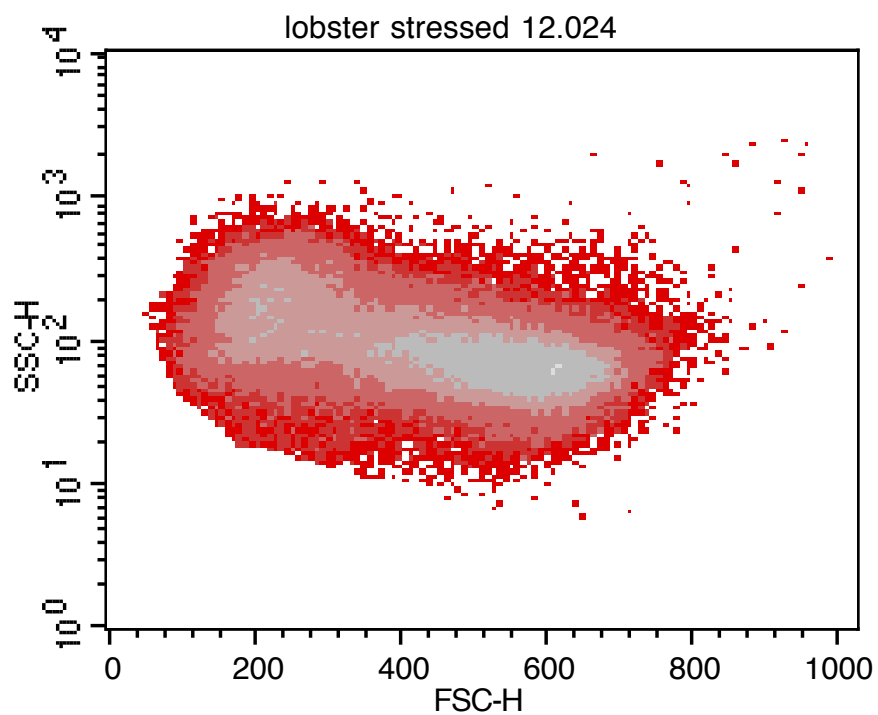


Figure 3.2. Density plot of *H. americanus* hemocytes based on size (FSC-H) and complexity (SSC-H).

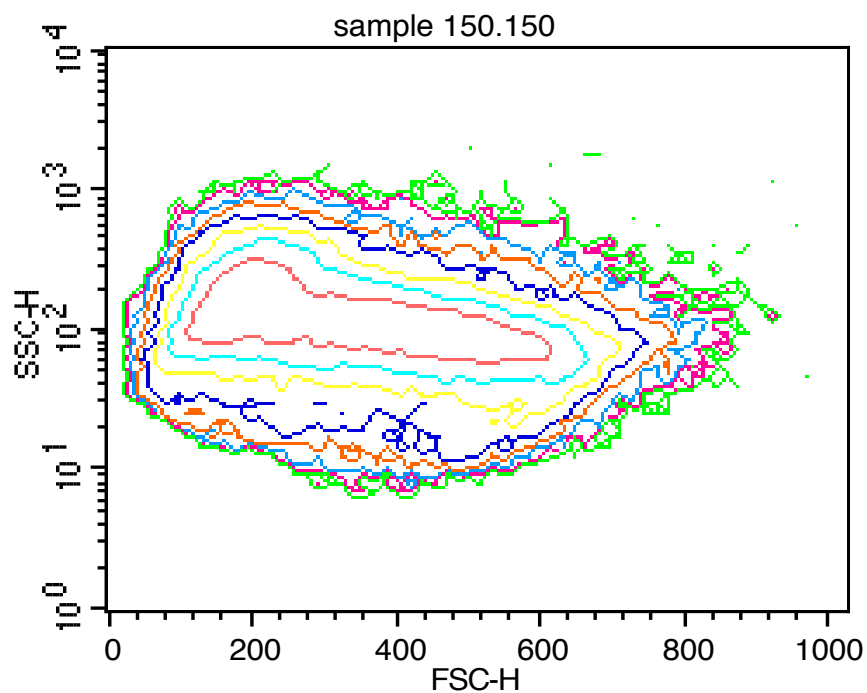


Figure 3.3. Contour plots of *H. americanus* hemocytes based on size (FSC-H) and complexity (SSC-H).

The total number of circulating hemocytes μl^{-1} of hemolymph was measured. However, the proportion of those total hemocytes made up by the different cell types were not recorded. There was no correlation between FACS hemocyte counts and hemocyte counts by microscopy ($r = 0.076$, $n = 104$, $p = 0.442$). No significant difference in total hemocyte counts was observed between “stressed” *H. americanus* sampled immediately post-capture, compared to the “acclimatized” lobsters kept in the system for 2 weeks post-capture prior to sampling (Man-Whitney: $Z = -1.413$, $p = 0.158$; Figure 3.4). There was no difference in total circulating hemocyte counts at 252 hours post dip treatments (ANOVA: $F_{3,55} = 0.765$, $p = 0.519$; Figure 3.5).

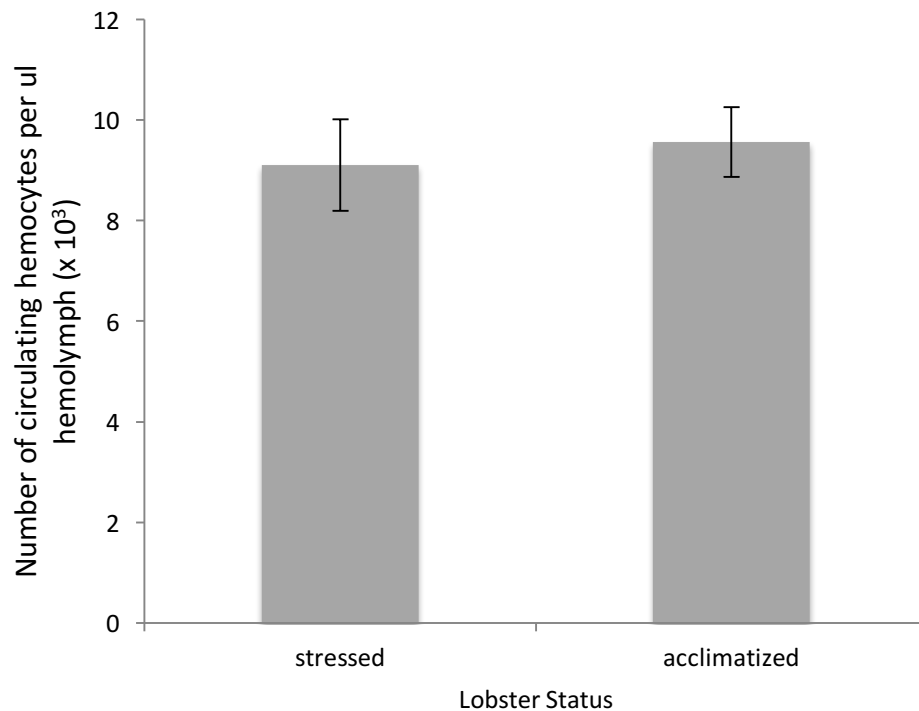


Figure 3.4. Total hemocyte counts μl^{-1} of hemolymph of “stressed” *Homarus americanus* immediately post-capture and lobsters “acclimatized” for 2 weeks post-capture, measured via flow cytometry.

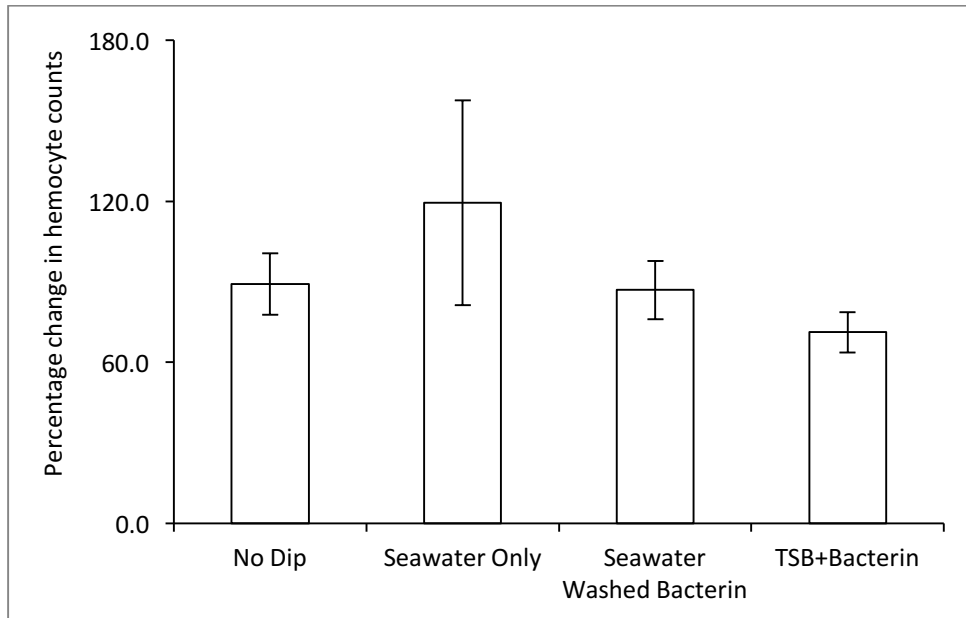


Figure 3.5. Percent change in total hemocyte counts μl^{-1} of hemolymph of lobsters at 252 hours post-dip treatments.

Respiratory Burst Activity

The respiratory burst activity was measured with zymosan particles in order to record the response of the hemocytes from the different treatment groups post-stimulation and phagocytosis of zymosan *in vitro*. There was no difference in the hemocyte respiratory burst activity at 252 hours post dip treatment of lobsters across all groups (ANOVA: $F_{3,35} = 0.974$, $p = 0.974$; Figure 3.6).

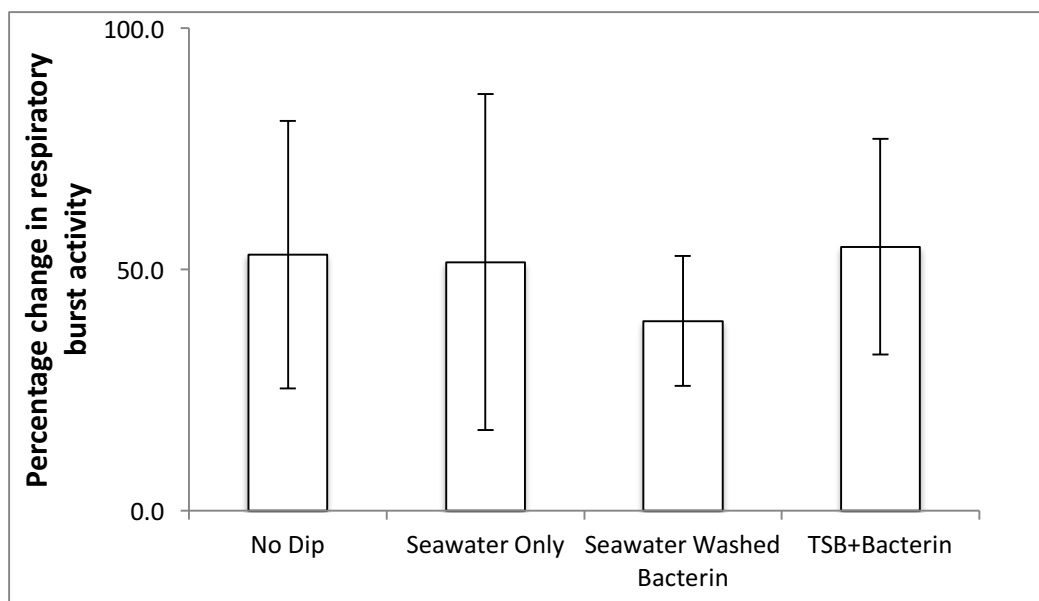


Figure 3.6. Percent change in respiratory burst activity of lobster hemocytes at 252 hours post dip treatments.

Discussion

This study utilized a *P. indicum* bacterin as an immunostimulant to stimulate the innate and cellular response of adult American lobsters. The immune response was evaluated by investigating total hemocyte count (THC) and the respiratory oxidative burst of hemocytes of lobsters exposed by immersion to a killed bacterin.

A detailed review of crustacean immune functions is reported in (Lin and Soderhall, 2011, Ellis et al., 2011, Hauton, 2012) and for *H. americanus*, in particular, (Bowden, 2017). In brief summary, the non-specific innate immune response in crustaceans is triggered by pattern recognition proteins (PRPs) in response to the pathogen associated molecular patterns (PAMPs) of bacterial peptidoglycans, lipopolysaccharides and fungal β -1,3 glucans (Hauton, 2012). The subsequent release of various effector molecules mobilizes circulating hemocytes to redistribute to the site of invasion where specific hemocytes degranulate producing reactive oxygen species,

antimicrobial peptides, lectins, proteinase inhibitors and opsonins that facilitate clotting, encapsulation, phagocytosis and melanization of foreign organisms (Moss and Allam, 2006, Lin and Soderhall, 2011, Clark et al., 2013). The degranulation, lysis and clumping of hemocytes results in a net reduction of circulating cells (hemocytopenia) (Lorenzon et al., 1999, Ridgway et al., 2006).

There have been numerous attempts to up-regulate the crustacean immune system. Sung *et al.* (1996), administered β 1, 3- and β 1,6-glucans to the tiger shrimp *Penaeus monodon*. Prebiotic immunostimulants such as mannan oligosaccharide (MOS) (Bio-Mos[®], Alltech, USA) were fed to larval and juvenile European lobsters *Homarus gammarus* (Daniels et al., 2010) and to juvenile tropical rock lobsters *Panulirus ornatus* (Sang and Fotedar, 2010). As previously discussed, other studies claim to have increased the survivability of impounded adult American lobsters with a bacterin produced from inactivated *Aerococcus viridans* var *homari* (Keith et al., 1992, Stewart et al., 2004). However, there does not appear to be a reproducible experiment that demonstrates effective immune “priming” beyond 72 hours (Smith et al., 2003, Hauton and Smith, 2007, Hauton et al., 2015), and to date, there is no unambiguous evidence of an adaptive immune response (immunological memory to specific antigens) in crustaceans.

Fluorescence-Activated Cell Sorting (FACS) with flow cytometry allows the user to plot individual cells based on their size and complexity (*i.e.* granularity). It is possible to differentiate cell population types based on these characteristics in teleost fish species, mammals, and birds., Researchers have been able to segregate subpopulations of live *H. americanus* hemocytes with flow cytometry (De Guise et al., 2005). However,

the prolonged maintenance of cell viability and the differentiation of hemocyte populations, via flow cytometry, were not possible during this project. The cell populations were too closely situated to gain accurate population limits, therefore, the total number of preserved circulating hemocytes μl^{-1} of hemolymph was measured. ((Hose et al., 1990) in their description of a decapod hemocyte classification scheme, concluded that morphology alone was inadequate to assign any cell to either hyaline cells or granulocyte category. (Battison et al., 2003) were able to differentiate up to 11 hemocyte cell types using a combination of nuclear and cytoplasmic characteristics but the authors also suggest that several of the cell types may represent precursors or a continuum of differentiation to maturity. Today the most frequently described crustacean hemocytes are grouped into hyaline, semigranular and granular but all cell types contain levels of granularity (Soderhall, 2016). Fixation of the hemocytes may have interfered with the ability to differentiate between size and granularity, but was necessary due to the large sample sizes.

The total circulating hemocyte counts for all groups remained relatively stable throughout the study for all treatment groups. No significant differences in hemocyte counts were observed in stressed lobsters versus acclimated lobsters. There was also no significant difference determined when comparing the percent change in the baseline total hemocyte counts versus any treatment group at the final sampling. Again, the hemocyte counts reflect the total circulating hemocytes and do not account for any aggregation/degranulation of hemocytes that might have occurred following presentation of the bacterin. It would be the natural function of the lobster to produce

and release new hemocytes from the hematopoietic tissue following the loss of recirculating hemocytes (Lin and Soderhall, 2011). It could also be exposure to the bacterin by immersion did not produce a measurable effect in regards to hemocyte count and a greater response may have been seen if the bacterin had been injected (Keith et al., 1992, Stewart et al., 2004). Of note, we saw no correlation between the FACS hemocyte counts and the hemocyte counts by microscopy. The counts achieved using the FACS were much higher than by microscopy of most samples compared. We hypothesize that the reasons for higher counts by FACS may be due to the FACS higher read volumes compared to the microscopy method. Also, there could be less clumping of cells in the FACS sampling process. Regardless of the final counts, the relative hemocyte concentrations measured by FACS is still comparable as they are measured by the same technique.

Respiratory burst is the rapid release of reactive oxygen species (superoxide radical and hydrogen peroxide) from different types of host immune cells to kill invading bacteria or fungi, and is conserved across invertebrate phyla. This activity was measured in *H. americanus* post-activation with zymosan particles in order to record the response of the hemocytes from the different treatment groups post-stimulation and phagocytosis of zymosan *in vitro*. While we did measure a respiratory burst activity in all groups, we did not observe a significant change in respiratory burst activity in any group when comparing the percent change in hemocyte respiratory burst activity from baseline sampling to the final time point of lobsters in all groups.

While this study did not demonstrate a significant change in measurable immune response through examining total circulating hemocytes and the respiratory burst activity of hemocytes, the study did demonstrate the safety of using the *P. indicum* bacterin. Future work should include comparing bacterin dose administration by immersion and injection. A bacterial challenge study with *P. indicum* to determine if a protective effect is achieved in lobsters post stimulation is also warranted. These are recommendations included in a newly proposed 'Minimum Information requested to support a Stimulant Assessment' for crustaceans (Hauton et al., 2015)

CHAPTER 4

THE INFLUENCE OF TEMPERATURE ON EPIZOOTIC SHELL DISEASE IN AMERICAN LOBSTERS (*HOMARUS AMERICANUS*)

Introduction

American lobsters, *Homarus americanus*, represent the most economically important fishery in the northwestern Atlantic Ocean. While currently the fishery remains viable and healthy in northern New England, lobster populations in southern New England (SNE) collapsed more than a decade ago, with no sign of recovery. The exact causes of the lobster population declines in SNE regions have yet to be determined but environmental, anthropogenic, and biological stressors and their connection with disease emergence must all be considered (Shields 2012). Of particular relevance, lobster population declines in SNE were coincident with increasing temperatures and the emergence of epizootic shell disease (ESD) indicating these as likely candidates contributing to population decline.

While the modest temperature increases along the Maine coast could be contributing to the current boom in lobster population growth in the Gulf of Maine and Georges Bank, temperatures in SNE appear to have surpassed the lobsters' thermal tolerance. Temperature records at a power station in Connecticut reveal the number of days in which water temperatures reached 20°C has increased dramatically over the past decade. In 1998 there were only five days over 20°C, whereas today 75 days per year or more breach 20°C. This thermal stress could not only impact lobster physiology directly, but also indirectly through increased disease susceptibility.

Epizootic shell disease emerged in the later part of 1996 in SNE and by 2000 was considered an epizootic that spread along the Northeastern coast. This new aggressive form of shell disease was described as a 'severe erosive shell disease affecting the dorsal carapace of *H. americanus*' (Smolowitz et al., 2005). The histological characteristics of the disease suggested a bacterial etiology but could not identify the bacteria. It is recognized as a significant disease syndrome and is described as having a multifactorial and complex etiology with increased temperatures implicated as a contributing factor (Castro et al., 2012, Shields, 2012). More recently, ESD has appeared with greater frequency in New England waters mainly south of Cape Cod. The last three years have seen a slight rise in the increase of ESD in Maine waters although prevalence still remains very low. Average annual prevalence of reported shell disease from Maine's lobster sea sampling program in 2003 was below 0.1% and in 2017 was reported at > 2.0% (<http://www.maine.gov/dmr/science-research/species/lobster/documents/2017monitoring.pdf>).

Culture-dependent and culture-independent bacterial investigations have been performed on shell diseased lobsters with the goal of determining the causative agent of ESD. This work has shown that the etiology of the disease is complex, likely involving multiple microbes. Genera of the *Altermonadaceae*, *Flavobacteriaceae*, *Pseudomonadaceae*, *Rhodobacteraceae* and *Vibrionaceae* families are well documented as dominant community members (Chistoserdov et al., 2005, Bell et al., 2012, Chistoserdov et al., 2012, Meres et al., 2012). Particular focus has been placed on two microbial genera consistently associated with ESD, *Aquamarina* and *Thalassobius*

species (Chistoserdov et al., 2012, Quinn et al., 2013, Quinn et al., 2017) but a true etiology has yet to be defined. In fact, the role of these bacteria in disease lesions remain unclear and a dysbiotic shift in the shell microbial community resulting from environmental stressors has been suggested (Meres et al., 2012). While past research focused on identification of microbial communities on wild caught or laboratory housed lobsters, these studies did not follow changes in microbial communities and profiles over time and temperature.

Current literature indicates that shell disease is not transferable in laboratory aquaria but that it will continue to progress in the individuals with shell disease. This study seeks to understand how seasonal temperature regimes influence antimicrobial activity and the progression of ESD in adult female American lobsters. Results from this work could provide critical information on the stability of the lobster shell's microbial community with increasing temperatures. Along with examining the progression of the disease, the antimicrobial activity of lobster hemolymph will be monitored over time and temperature. We hypothesize that more extreme temperature regimes will exacerbate ESD progression, reduce metrics of immune health, and alter microbial communities.

Methods

System Design and Function

The project consisted of maintaining aquaria tank systems at three temperature regimes that compare to recorded seasonal ocean temperatures for Southern New England (SNE), Southern Maine (SME) and Northern Maine (NME) regions (Figure 4.1.).

Average monthly temperatures were obtained through NOAA's National Oceanographic Data Center (NODC, 2013).

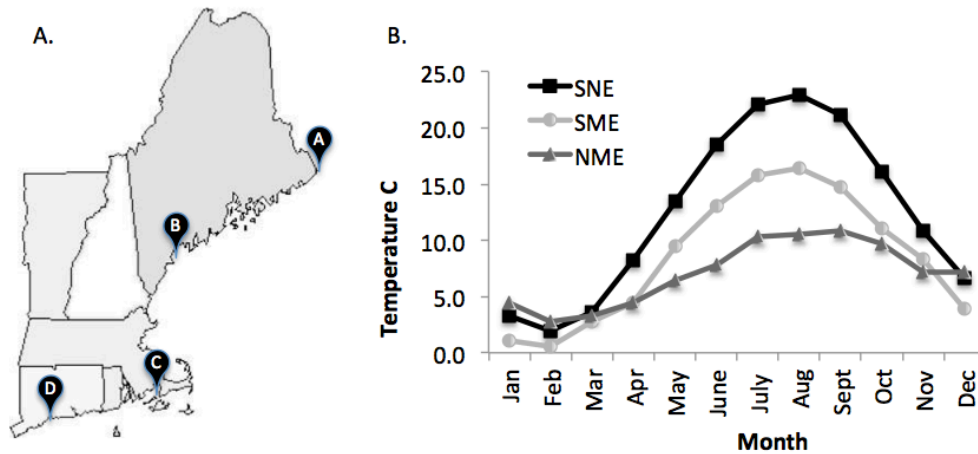
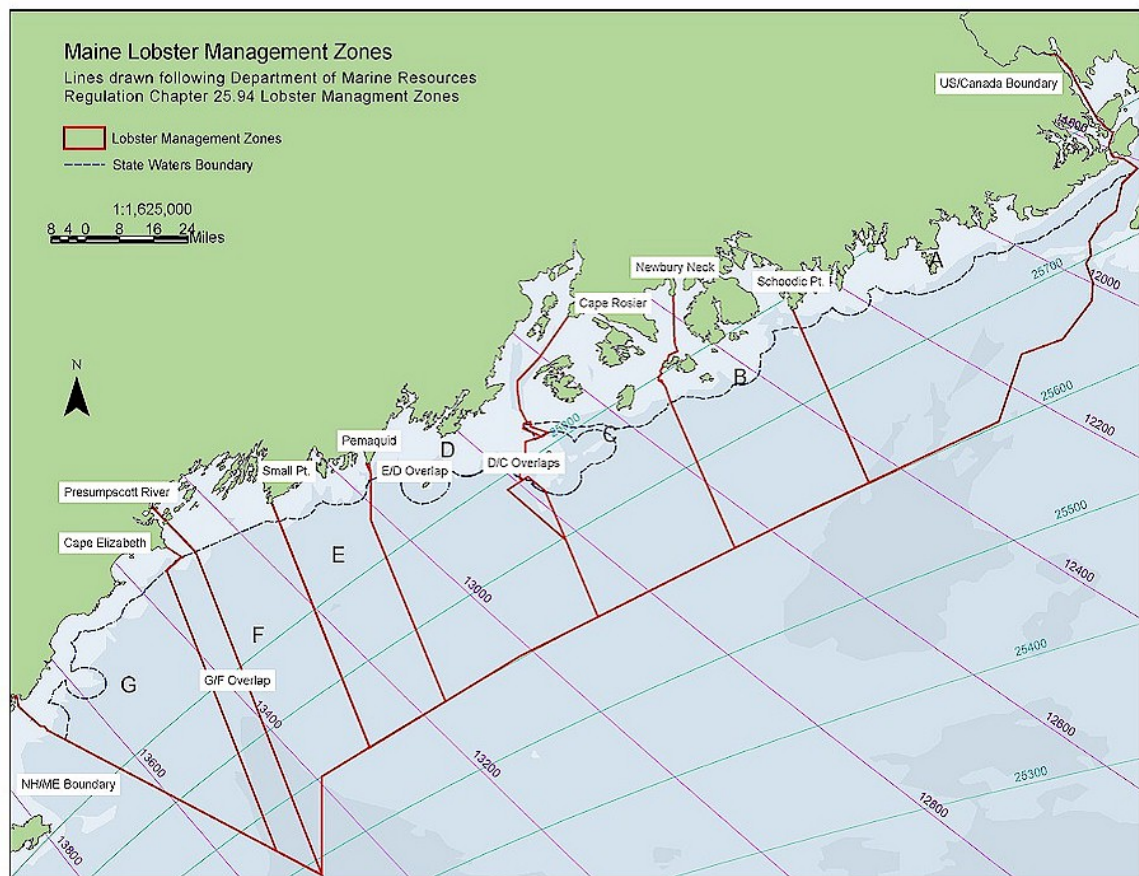


Figure 4.1. Water temperature regimes. A. Temperatures were obtained through the National Oceanographic Data Center (NODC). NODC temperatures reflect those recorded near Eastport, ME (A); Portland, ME (B); and an average of temperatures from Woods Hole, MA (C) and New Haven, CT (D) was used to represent Southern New England. B. Annual temperature cycles used in this project to represent Southern New England (SNE), Southern Maine (SME) and Northern Maine (NME).

The aquaria systems consisted of three individual 1400 L recirculating artificial sea water units with two 365 L tanks in each unit. For each system, water was mechanically and chemically filtered and passed through a bank of two 65 W ultraviolet sterilizers before flowing back into the lobster holding tanks. Daily water quality was performed that consisted of monitoring temperatures and dissolved oxygen. Levels of ammonia, nitrogen and pH were tested twice a week and 25% water changes performed twice a week. Water quality levels were maintained at levels of total ammonia and nitrogen less than 1.0 mg/L, nitrite, less than 0.1 mg/L and a pH of 8.0 to 8.1, salinity, 33–35 ppt, and oxygen, > 7.0 mg/L. Initially, all tank systems were held at 11 ± 2 °C for temperature.

Lobsters

Fifty-seven female lobsters, were collected during the Maine State ventless trap survey in late June 2016. All lobsters were collected from Maine's lobster management zones F and G (Figure 4.2).



C. Rubicam, 8/9/02, DMR Maine Whale Plan

Figure 4.2. Maine lobster management zones.

Non-shell diseased lobsters (no apparent shell disease) ($n = 28$) and shell diseased lobsters (apparent signs of ESD; $n=29$) were randomly distributed between the three systems, therefore each system held a minimum of nine non-shell diseased (referred to as healthy) and nine shell diseased lobsters (referred to as diseased). Healthy versus diseased lobsters were held in separate tanks within the system. Lobsters were individually compartmentalized to reduce agonistic behavior and cannibalism, un-

banded to allow for normal grooming, and fed previously frozen fish, shrimp and green crabs throughout the study. Lobsters were acclimated for approximately three weeks before seasonal temperature regimes were initiated. The three systems were phased in to current seasonal temperatures at no more than 2°C per day. Three weeks allowed for lobsters to acclimate to laboratory aquaria systems, eliminating stress response from capture and transport and allowed lobsters to come to a 'resting' state (Basti et al., 2010a). Lobsters were observed over 11 months with reflecting temperatures of a full year of seasonal temperature patterns.

Molt Stage Evaluation

The relative molt stage of lobsters was determined by estimating shell rigidity (Aiken 1980) and grouped into postmolt (stages A, B, C1 and C2); intermolt (stages C3 and C4); and premolt (stage D).

Shell Disease Evaluation

The extent of shell disease on lobsters was accessed using the shell disease index of: 0, no observable signs of disease; 1+, shell disease signs on 1-10% of the shell surface; 2+, shell disease signs on 11-50% of the shell surface; 3+, shell disease signs on > 50% of the shell surface (Smolowitz et al., 2005).

Carapace Sampling for Microbial Analyses

Carapace sampling was performed by using a sterile cotton tipped applicator and swabbing an approximate 4 cm² square surface area of the dorsolateral region of the cephalothorax.



Figure 4-3. Lobster carapace sampling

Each lobster was sampled in the same anatomical location at each sampling time point. The right side of the dorsolateral area of the cephalothorax was sampled for the baseline sampling, the left side for the Time 1, and the right side again for Time 2. The applicator was repeatedly rolled over the approximate 4 cm² square surface area for 30 seconds and then placed in a tube containing 0.9 mls of sterile artificial sea water (ASW). These samples were held on ice and then processed for microbial community sampling.

Hemolymph Sampling

Approximately 1.5 mls of hemolymph were collected aseptically from the dorsal abdominal artery into a sterile 3.0 cc syringe with a 23-gauge needle and then discharged into a 2 ml microfuge tube. Microfuge tubes were held on ice and centrifuged at 10,000g within 10 minutes of sample collection to avoid coagulation of the hemolymph. The plasma (cell free hemolymph) was then transferred to a new microfuge tube. All plasma samples were stored at -80C until the anti-microbial assay was performed.

Lobster Plasma Antimicrobial Assay

The antimicrobial activity of the plasma (hemolymph with hemocytes removed) was measured using a modified turbidimetric assay adapted from (Noga et al., 1994) where *Escherichia coli* D31 (Monner et al., 1971) is treated with plasma, and bacterial growth is compared among treatments after incubation. The bacterium *E. coli* D31 was acquired from Yale University's bacterial culture collection. The stored frozen lobster plasma samples were thawed on ice and filter sterilized using a 0.22 µm filter prior to testing. The procedure was performed as described in (Homerding et al., 2012) but was modified by performing the entire assay in a 96 well plate. For each plasma sample, 10 µl of the filter sterilized plasma was incubated with 10 µl of the prepared bacterial suspension and 30 µl of PBS for 30 minutes at room temperature. Next, 450 µl of cold Trypticase Soy Broth containing additional 1% NaCl and 0.1mg/L streptomycin was added. Each plasma sample was then immediately plated in triplicate onto a 96 well plate (Falcon). Microbial growth was measured by absorbance at 570nm on a Biotek plate reader every 4 hour for 22 hours. Antimicrobial activity, the bacterial growth inhibition, of plasma was calculated as the mean optical density and compared to the *E.coli* D31 growth control without lobster plasma. Increasing optical densities (OD) indicate increasing bacterial growth.

Microbial Enumeration and Culture

The swab placed in 0.9 mls of sterile ASW was vortexed for 30 seconds to dislodge cellular and microbial material. The swab was aseptically removed and discarded. This sample containing the dislodged material was considered the undiluted

sample for the microbial enumeration and culture assays. A serial dilution was performed out to 10^{-3} for the baseline and Time 1 and to 10^{-5} for Time 2 by serially transferring 100 μ l into 900 μ l of sterile ASW. All dilutions were plated on 2 media by adding 100 μ l to each plate and spreading using a flame sterilized glass spreader. The two medias used were Trypticase Soy Agar with 5% sheep red blood cells +1.5% NaCl (BA) (Northeast Laboratory, Waterville, ME. USA), and a Marine Sea Salt Agar plate (MA), prepared in house with 10.0 g Trypticase Soy Agar (Becton, Dickinson and Co. Sparks, MD. USA), 20.0g agar (Becton, Dickinson and Co. Sparks, MD. USA), 33.8g instant ocean (Crystal Sea® Marinemix, Baltimore, MD), 1000 ml deionized water, and then autoclaved for sterility. All inoculated plates were incubated at 15 ± 2 °C for 10 days. Ten days allowed for maximum growth of all bacterial types and allowed for a greater degree of colony morphology differentiation. Culturable microbial growth was enumerated by standard plate count methods and predominant colonies selected per media type for phenotypic identification using the Biolog® Microbial Identification System Gen II (BMIS) and Sanger 16S rRNA sequencing on selected isolates. The five most predominant colonies on each media type were selected for identification. Isolated colonies were re-streaked onto the corresponding medium for re-isolation and purity and the isolates incubated again for 48-72 hours at 16°C. Preliminary biochemical profiling included Gram stain, oxidase, catalase reaction and carbohydrate utilization and H₂S production with triple sugar iron (TSI) medium. Isolates were then filtered according to preliminary similar biochemical reactions prior to final identification using

the BMIS. Those isolates that could not be identified by BMIS were identified using 16S rRNA sequencing.

Statistical Analyses

Lobster weights and mortality: To assess differences in baseline weights of non-shelled diseased and shell diseased lobsters, weights were compared using a *t* test. Growth of the lobsters during the experiment was assessed by comparing the lobster weight at the baseline sampling to weight at the third sampling using a paired *t*-test. In addition the percentage growth for each lobster was calculated, then growth of non-shell disease lobsters was compared to shell diseased lobsters. The total number of mortalities throughout the experiment from non-shell diseased and shell diseased lobsters were compared for each temperature regime (i.e. SNE, SME, and NME) using a Fisher's exact tests.

Antimicrobial activity: The mean optical density readings at 22 hours for each treatment were compared with ANOVAs.

Microbial community sampling: At the baseline sampling, the bacterial loads of non-shell diseased and shell diseased lobsters were compared with a *t* test. For each sampling period, the bacterial loads within the non-shell disease and shell disease were compared across time points (ie. baseline, lower and higher seasonal temperatures) using Kruskal-Wallis tests. Then the bacterial loads between non-shell disease and shell disease lobsters at each temperature regime (i.e. SNE, SME, and NME) were compared with Mann-Whitney tests. Shannon-Weiner (H') equitability index was used to assess the diversity in the bacterial communities for all treatment groups using the following

formula, where N is the total number of species found and n_1 is the number of individuals of one particular species found (Beisel and Moreteau, 1997):

$$H' = - \sum \left[\left(\frac{n_1}{N} \right) \ln \left(\frac{n_1}{N} \right) \right]$$

Antimicrobial assay analyses were conducted using GraphPad Prism v. 7 and all analyses were conducted using SPSS v. 24.0. Nonparametric tests were used where the data were not normally distributed.

Results

System Design and Function

The seasonal temperature profiles for each system were met, where System 1 (Low Temp) was aimed at targeting NME, System 2 (Mid Temp) at SME and System 3 (High Temp) at SNE (Figure 4.4).

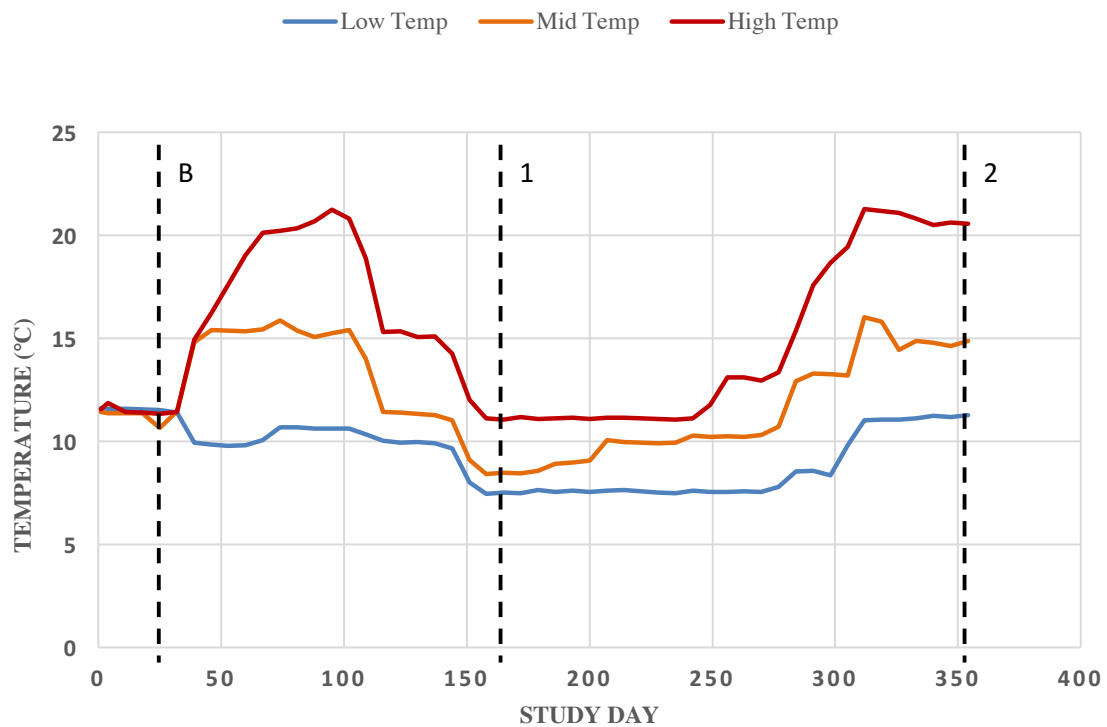


Figure 4-4. Average daily system temperatures for each of the three temperature regimes over the course of the experiment. The dotted lines indicate approximate sampling periods (B = Baseline; 1 = First sampling; 2 = Second sampling).

Based on the start date of this study, the temperatures began with mid-summer temperatures. In order to cycle through all seasonal temperature profiles, the seasonal temperatures were minimally accelerated so that the study went from summer temperatures through a full season and ended with peak summer temperatures of a second season. Figure 4.3 also indicates lobster sampling times.

Lobster Collection and Husbandry

Tables 4.1. and 4.2. present the status of the lobster in regards to shell hardness and shell disease index at each sampling time point.

Table 4.1. Shell hardness scores and shell disease scores for all non-shell diseased lobsters across all three temperature regimes. The “-” indicates death of the lobster or missing data and * indicates molt. Low, Mid, and High reflects NME, SME and SNE reflectively. Shell hardness columns are 1= C1, 2= C2, 3=C3, 4=C4 and 5=D, and Shell disease index columns are 0= no apparent signs of ESD, 1= 1+ ESD, 2 = 2+ ESD, 3 = 3+ ESD.

Lobster	Temp	Tank	Shell Hardness			Shell Disease Index		
			Baseline	First Sampling	Second Sampling	Baseline	First Sampling	Second Sampling
6811	Low	1	4	*3	3	0	0	0
6812	Low	1	4	*2	3	0	0	0
6813	Low	1	4	*2	2	0	0	0
6818	Low	1	5	*3	4	0	0	0
6819	Low	1	4	4	4	0	0	0
6820	Low	1	5	-	-	0	-	-
6821	Low	1	1	3	4	0	0	0
6822	Low	1	2	*3	3	0	0	0
6828	Low	1	5	*3	4	0	0	0
6823	Mid	4	4	*2	3	0	0	0
6843	Mid	4	4	4	4	0	0	0
6844	Mid	4	4	4	0	0	0	-
6845	Mid	4	5	*2	3	0	0	0
6846	Mid	4	5	4	5	0	0	0
6847	Mid	4	4	*3	4	0	0	0
6848	Mid	4	4	4	3	0	0	0
6849	Mid	4	4	*2	2	0	0	0
6850	Mid	4	5	4	4	0	0	0
6831	High	5	4	*2	2	0	0	0
6832	High	5	*2	2	3	0	0	0
6833	High	5	4	4	5	0	0	0
6834	High	5	3	3	3	0	0	0
6835	High	5	4	*3	*2	0	0	0
6836	High	5	2	2	2	0	0	0
6837	High	5	5	*2	2	0	0	0
6838	High	5	*1	-	-	0	-	-
6839	High	5	*5	-	-	0	-	-
6840	High	5	*1	2	3	0	0	0

Table 4.2. Shell hardness scores and shell disease scores for all shell diseased lobsters across all three temperature regimes. The “–” indicates death of the lobster or missing data and * indicates molt. Low, Mid, and High reflects NME, SME and SNE reflectively. Shell hardness columns are 1= C1, 2= C2, 3=C3, 4=C4 and 5=D, and Shell disease index columns are 0= no apparent signs of ESD, 1= 1+ ESD, 2 = 2+ ESD, 3 = 3+ ESD.

Lobster	Temp	Tank	Shell Hardness			Shell Disease Index		
			Baseline	First Sampling	Second Sampling	Baseline	First Sampling	Second Sampling
6827	Low	2	3	*2	2	1	0	0
6876	Low	2	5	-	-	3	-	-
6877	Low	2	5	*1	2	1	0	0
6878	Low	2	5	4	4	1	1	1
6879	Low	2	4	4	5	1	1	1
6882	Low	2	5	*2	5	1	0	0
6883	Low	2	-	2	4	-	0	0
6888	Low	2	5	-	-	1	-	-
6889	Low	2	5	*2	5	1	0	0
6890	Low	2	4	4	5	1	1	1
6766	Mid	3	*1	3	1	0	1	1
6767	Mid	3	5	*1	2	1	0	0
6768	Mid	3	5	*3	4	2	0	1
6769	Mid	3	5	-	-	2	-	-
6770	Mid	3	5	*1	2	2	0	0
6824	Mid	3	-	3	-	-	0	-
6826	Mid	3	2	3	-	0	0	-
6873	Mid	3	4	-	-	1	-	-
6874	Mid	3	5	*2	2	3	1	1
6875	Mid	3	5	-	-	2	-	-
6761	High	6	4	*2	3	1	0	0
6762	High	6	5	*2	2	3	0	0
6763	High	6	4	4	-	1	2	-
6764	High	6	5	*2	3	3	0	1
6765	High	6	5	*2	3	2	0	0
6825	High	6	5*	-	-	2	-	-
6871	High	6	5*	-	-	1	-	-
6872	High	6	5	*1	-	3	0	-
6881	High	6	5	*2	2	3	0	1

At the Baseline sampling, based on shell hardness, the molt stages of the healthy lobsters were 8 (29%) premolt, 14 (50%) intermolt and 6 (21%) postmolt. The molt stages of the diseased lobsters were 19 (66%) premolt, 6 (20%) intermolt and 4 (14%) postmolt. For the diseased lobsters, at the baseline sampling, shell disease evaluation indicated that 6 (21%) of the lobsters were rated as 3+, 6 (21%) as 2+, 13 (44%) as 1+ and 4 (14%) at 0 (these lobsters molted during acclimation before the Baseline sampling was performed). Pictures displaying various shell disease indices are represented in Figure 4.5. Seventeen of the 28 healthy lobsters (61%) molted and observationally all 28 lobsters demonstrated no signs of shell disease for the duration of the study. One healthy lobster in System 3 molted twice during the study. Seventeen of the 29 diseased lobsters (59%) molted during the study. Four of the 17 diseased lobsters that molted developed signs of shell disease post molt; two lobsters from the mid-range temperature system and 2 from the high temperature system. Observationally, the shell disease index remained the same or progressed minimally during the study.

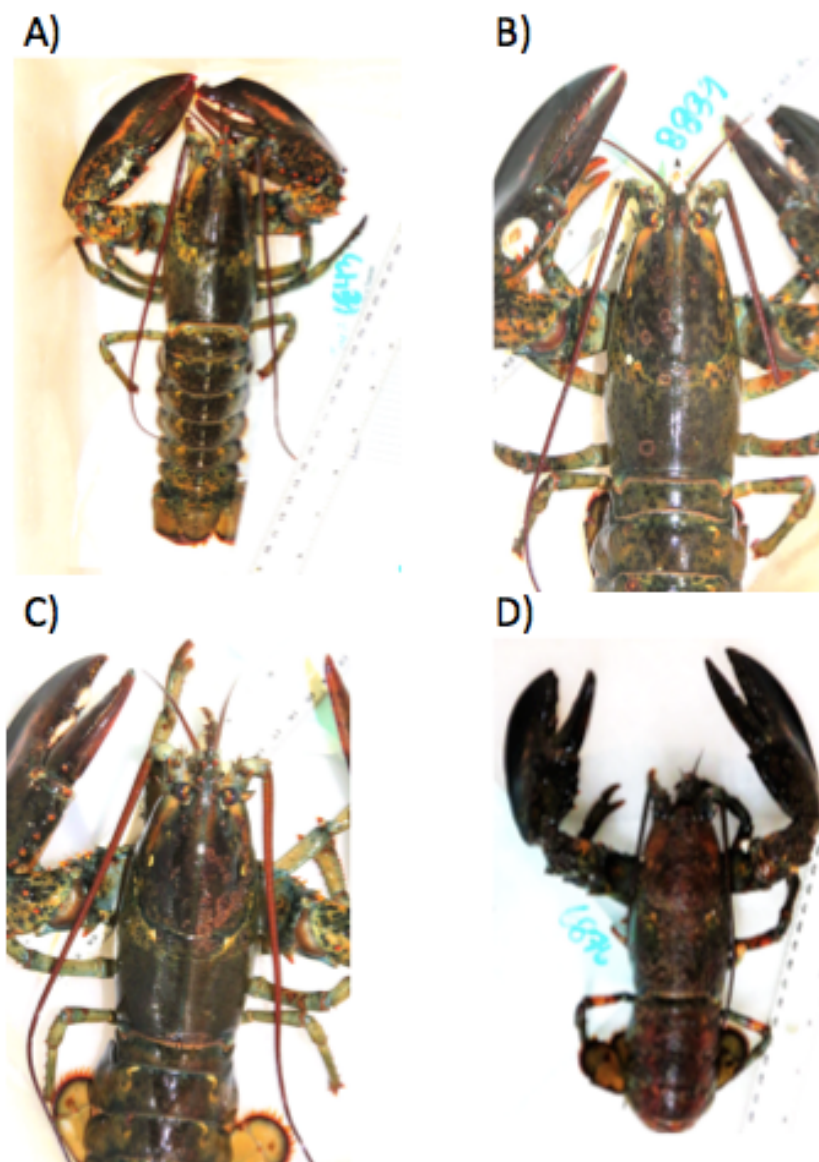


Figure 4-5. Examples of shell disease indices: A) 0, no observable signs of disease, B) 1+, shell disease signs on 1-10% of the shell surface, C) 2+, shell disease signs on 11-50% of the shell surface, D) 3+, shell disease signs on > 50% of the shell surface

Lobster growth was monitored by weight gain at each sampling time point. At the baseline sampling, there was no difference in weight between healthy lobsters and diseased lobsters (t test: $t_{53} = -1.675$, $P = 0.100$) (Figure 4.6).

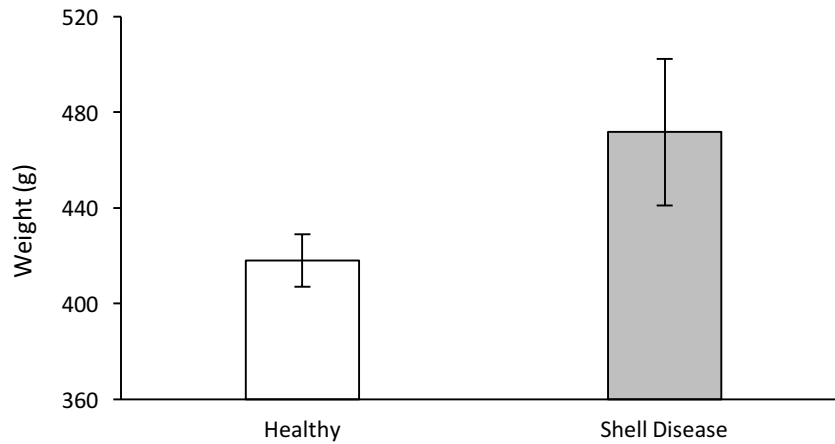


Figure 4-6. The average weight of non-shell diseased and shell diseased lobsters at the baseline sampling. Error bars indicate the standard errors.

There was a significant increase in weight for healthy and diseased lobsters across the baseline and last sampling periods (healthy lobsters: paired t test: $t_{24} = -6.086$, $P < 0.001$; shell diseased lobsters: paired t test: $t_{16} = -4.328$, $P = 0.001$; Figure 4.7) indicating lobster growth during the study duration. Non-shell diseased lobsters grew on average 13.9% of their original body weight (range = -9 to 30%) and shell diseased lobsters grew on average 10.2% of the original body weight (range = -11 to 22%).

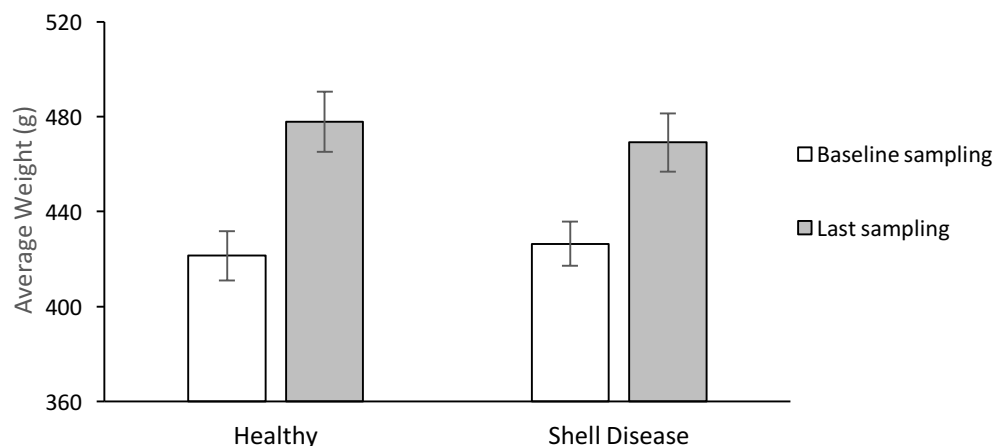


Figure 4-7. Average weights for non-shell diseased and diseased lobsters for the baseline and last sampling periods. Error bars indicate standard error.

There was no difference in the number of mortalities between healthy and shell diseased in the SNE (i.e. warmest) temperature regimes (NME: $\chi^2 = 0.28$, $p = 0.60$; SNE $\chi^2 = 0.25$, $p = 0.25$; Figure 4.8.). There is a trend for the shell diseased lobsters to have higher mortality than non-shell diseased in SME temperature regime ($\chi^2 = 3.32$, $p = 0.07$; Figure 4.8).

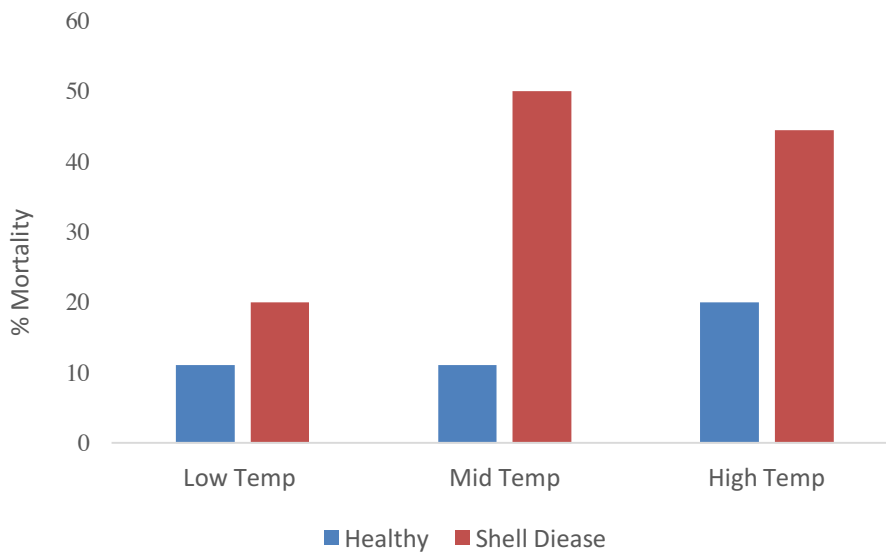


Figure 4-8. Percent mortality during the course of the study.

Antimicrobial Assay

Results from the baseline testing indicated that there was no bacterial growth inhibition from any samples and therefore no differences between the antimicrobial activity of plasma collected from healthy and diseased lobsters at 12.5°C (ANOVA: $F_{2,47} = 1.428$, $p = 0.25$; Figure 4.9). For sampling time 1 (i.e. winter sampling) again there was no demonstrated bacterial growth inhibition and the antimicrobial activity of plasma from healthy lobsters was not affected by temperature at 7.5°C, 8.5°C, and 11.0°C (ANOVA: $F_{3,23} = 1.56$, $p = 0.23$; Figure 4.10).

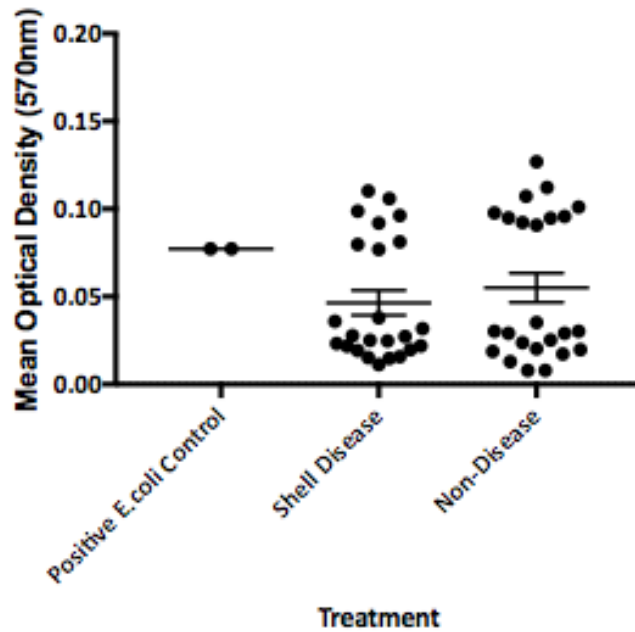


Figure 4-9. Baseline antimicrobial activity of plasma from healthy and shell diseased lobsters. Lobsters held at a temperature of 12.5°C compared to the *E.coli* D31 positive control after 22 hours of incubation. Error bars are standard error.

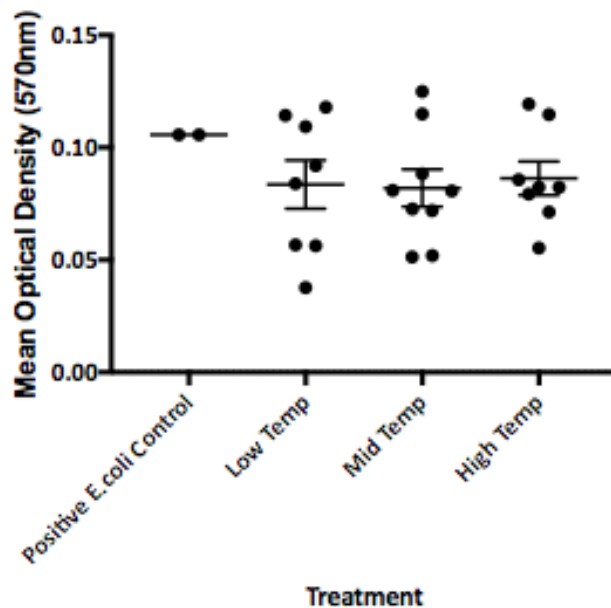


Figure 4-10. Time 1 antimicrobial activity of plasma from healthy lobsters held at varying temperatures. Results compared to the *E.coli* D31 positive control after 22 hours of incubation. Error bars are standard error.

There was also no bacterial growth inhibition and the antimicrobial activity of plasma observed from diseased lobsters was also not affected by temperature at 7.5°C, 8.5°C, and 11.0°C (ANOVA: $F_{3,18} = 1.083$, $p = 0.468$; Figure 4.11). When comparing the antimicrobial activity of plasma collected from pooled healthy and diseased lobsters across temperatures 7.5°C, 8.5°C, and 11.0°C, there were no differences (ANOVA: $F_{2,44} = 1.261$, $p = 0.29$; Figure 4.12).

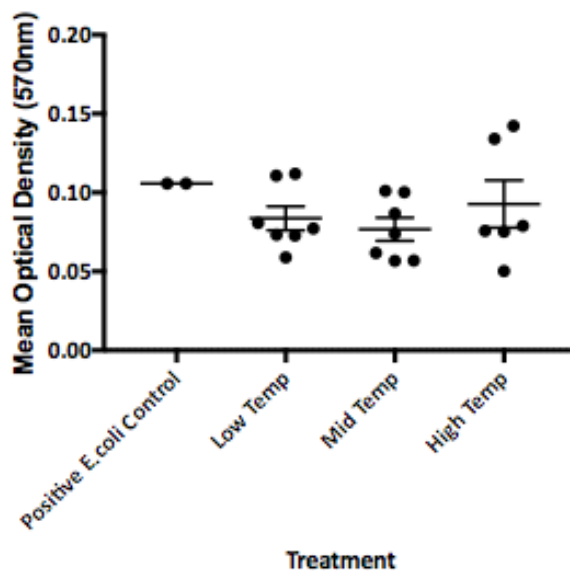


Figure 4-11. Time 1 antimicrobial activity of plasma from shell diseased lobsters at varying temperatures. Results compared to the *E.coli* D31 positive control after 22 hours of incubation. Error bars are standard error.

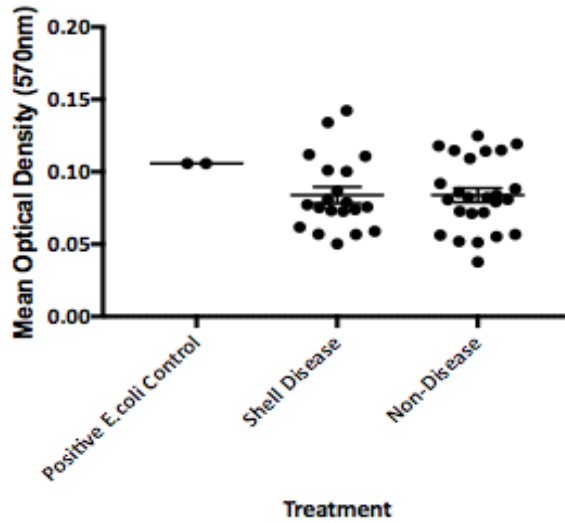


Figure 4-12. Time 1 antimicrobial activity of plasma from healthy and shell diseased lobsters at varying temperatures. Results compared to the *E.coli* D31 positive control after 22 hours of incubation. Error bars are standard error.

For Time 2, summer sampling, the antimicrobial activity of plasma from healthy lobsters was not affected by temperature at 10.0°C, 15°C, and 21°C (ANOVA: $F_{3,23} = 0.0612$, $p = 0.98$; Figure 4.13). Results of antimicrobial activity of plasma from diseased lobsters was also not affected by temperature at 10.0°C, 15°C, and 21°C (ANOVA: $F_{3,16} = 0.897$, $p = 0.46$; Figure 4.14). When comparing the pooled antimicrobial activity of plasma collected from healthy and diseased lobsters across temperatures 10.0°C, 15°C, and 21°C there were no differences (ANOVA: $F_{2,42} = 0.0582$, $p = 0.94$; Figure 4.15).

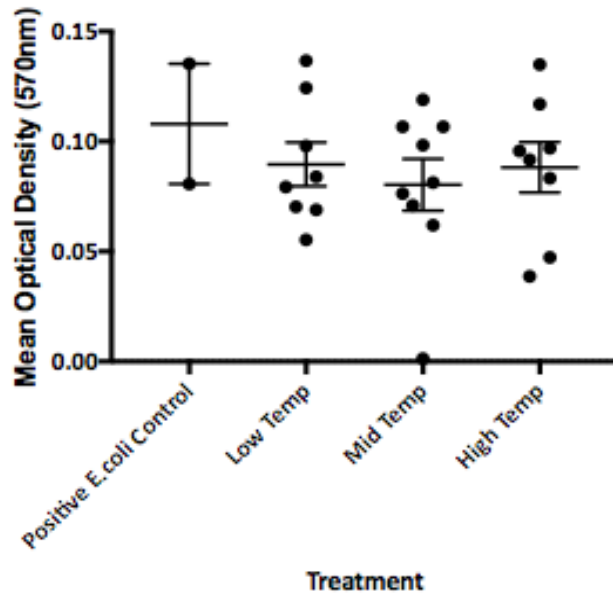


Figure 4-13. Time 2 antimicrobial activity of plasma from healthy lobsters held at varying temperatures. Results compared to the *E.coli* D31 positive control after 22 hours of incubation. Error bars are standard error.

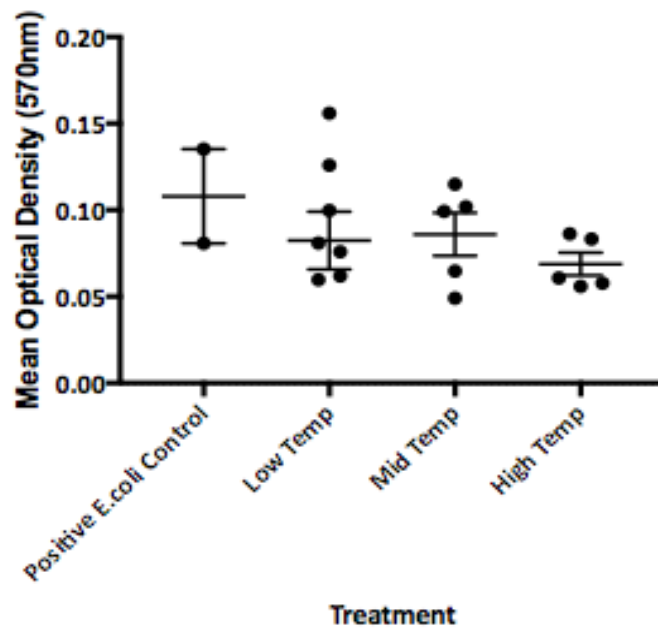


Figure 4-14. Time 2 antimicrobial activity of plasma from shell diseased lobsters at varying temperatures. Results compared to the *E.coli* D31 positive control after 22 hours of incubation. Error bars are standard error.

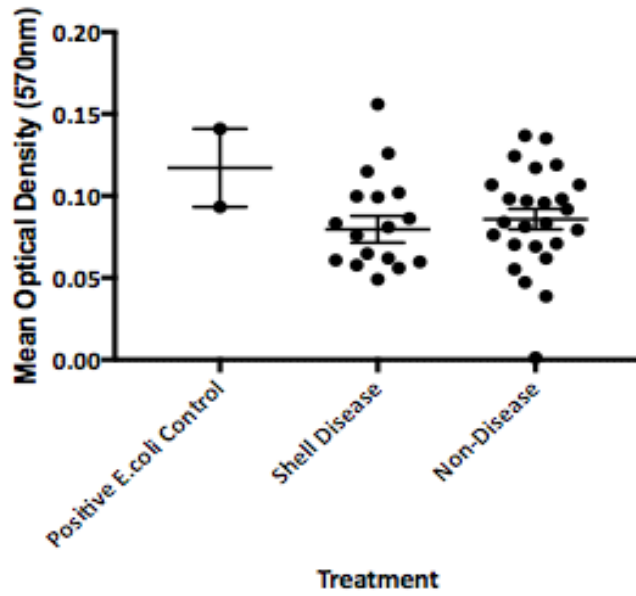


Figure 4-15. Time 2 antimicrobial activity of plasma from healthy and shell diseased lobsters at varying temperatures. Results compared to the *E.coli* D31 positive control after 22 hours of incubation. Error bars are standard error.

Microbial Community Sampling

Over the three sampling time points, a total of 145 carapace samples were processed for microbial enumeration and culture. At the baseline sampling the bacterial counts for lobsters from all three systems were combined as the temperatures were all the same (temp = 12.5 C). Lobsters showing signs of shell disease had significantly more bacteria per cm² than non-shell diseased lobsters (*t* test: $t_{48} = -1.989$; $P = 0.052$; Figure 4.16).

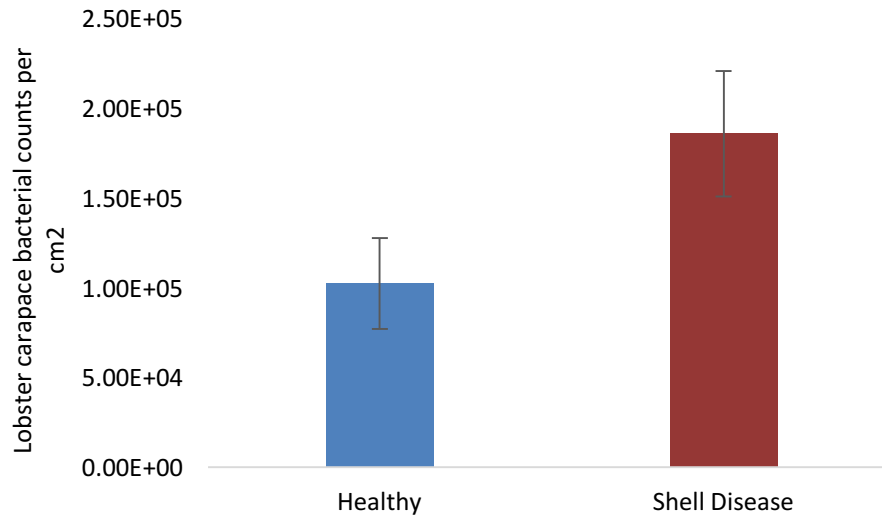


Figure 4-16. Baseline, bacterial counts plated on marine agar plates with a temperature of 12.5°C on average for all three systems. Error bars indicate the standard error.

At the first sampling period, for healthy and diseased lobsters, there were significantly more bacteria in the high temperature regimes than the low and mid temperature regimes (Kruskal-Wallis: non-shell diseased: $\chi^2 = 14.385$, $df = 2$, $P = 0.001$; shell diseased: $\chi^2 = 7.232$, $df = 2$, $P = 0.027$; Figure 4.17). In the low temperature regime the shell diseased lobsters had significantly more bacteria than the healthy lobsters (Mann-Whitney: $Z = -2.100$, $P = 0.036$, Figure 4.17). There was no difference in bacterial counts between healthy and diseased lobsters in the mid and high temperature regime (Mann-Whitney: mid temperature regime: $Z = -0.476$, $P = 0.634$; high temperature regime: $Z = -0.880$, $P = 0.379$; Figure 4.17).

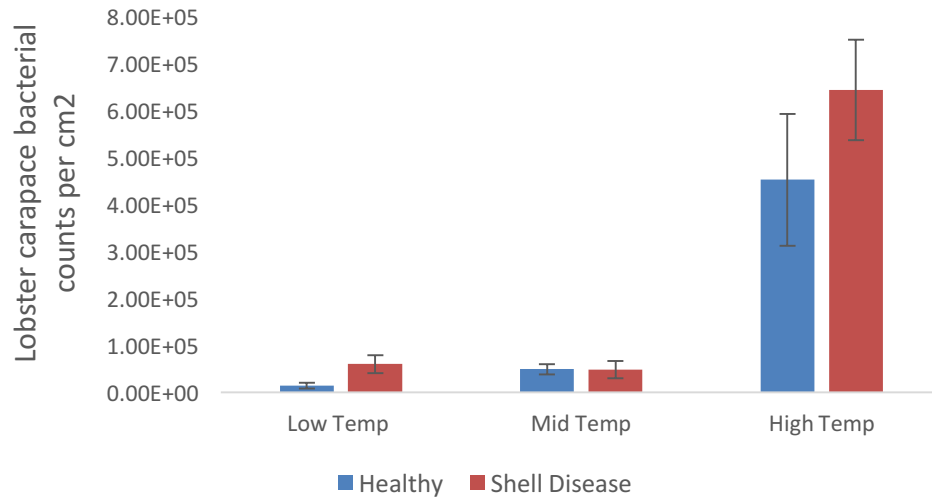


Figure 4-17. Time 1, bacterial counts plated on marine agar plates. With the average system temperatures being: Low temp = 7.5°C, Mid temperature = 8.5°C, and High temp = 11.0°C. Error bars indicate standard error.

At the second sampling period, there were significantly more bacteria for both healthy and diseased lobsters. There were significantly more bacteria in the mid and high temperature regimes than the low temperature regimes (Kruskal-Wallis: Healthy: $\chi^2=13.412$, df = 2, P = 0.001; Diseased: $\chi^2=11.498$, df = 2, P = 0.003; Figure 4.18). In the low temperature regime, there was a trend for the diseased lobsters to have more bacteria than the healthy lobsters (Mann-Whitney: Z = -1.890, P = 0.059, Figure 4.18). There was no difference in bacterial counts between healthy and diseased lobsters in the mid and high temperature regime (Mann-Whitney: mid temperature regime: Z = -0.867, P = 0.386; high temperature regime: Z = -1.757, P = 0.079; Figure 4.18).

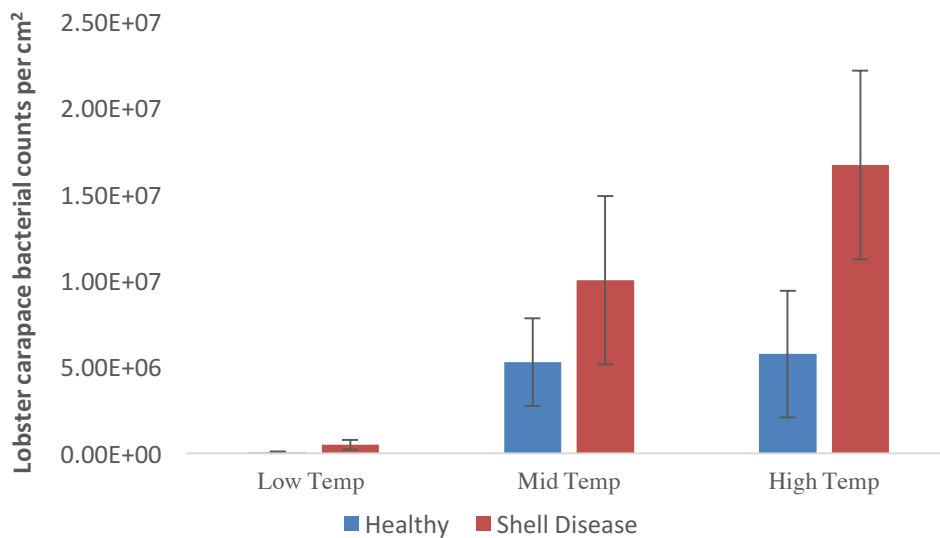


Figure 4-18. Time 2, bacterial counts plated on marine agar plates. With the average system temperatures being: Low temp = 10°C, Mid temperature = 15°C, and High temp = 21.0°C. Error bars indicate standard error.

Using the two media over 1,450 isolates were identified by phenotypic identification and/or 16sRNA sequencing. The purpose of this work was to determine the microbial ‘stability’ as in changes to community profile over time and temperature. For review, BMIS provides identification by means of phenotypic/biochemical characteristics. BMIS results range in probability of identification and can provide high probability to the species level while some results provide a genus with moderate to low probability of species and others to genus only. For the purpose of this study, results are evaluated to genus level only for community profiling. Table 4.3 presents the 4 phyla, 20 families and the top 36 genera of bacteria identified for this study, inclusive of the genera most frequently associated with ESD such as *Aquamarina*, *Flavobacterium*, *Polaribacter*, *Sulfitobacter*, *Psuedoaltermonas*. Our study did not identify the presence of *Thalassobious* species using culture dependent techniques.

Table 4.3. Taxonomy of lobster shell bacterial isolates

Phylum	Class	Order	Family	Genus
Actinobacteria	<i>Actinobacteria</i> <i>Actinomycetales</i>	<i>Actinomycetales</i>	<i>Gordoniaceae</i>	<i>Gordonia</i>
			<i>Intrasporangiaceae</i>	<i>Kytococcus</i>
			<i>Tsukamurellaceae</i>	<i>Tsukamurella</i>
		<i>Corynebacterineae</i>	<i>Corynebacteriaceae</i>	<i>Corynebacterium</i>
			<i>Nocardiaceae</i>	<i>Rhodococcus</i>
		<i>Micrococcales</i>	<i>Micrococcaceae</i>	<i>Micrococcus</i>
		<i>Micrococcineae</i>	<i>Microbacteriaceae</i>	<i>Curtobacterium</i>
				<i>Microbacterium</i>
Bacteroidetes	<i>Flavobacteria</i>	<i>Flavobacteriales</i>	<i>Flavobacteriaceae</i>	<i>Aquimarina</i>
				<i>Chryseobacterium</i>
				<i>Empedobacter</i>
				<i>Flavobacterium</i>
				<i>Formosa</i>
				<i>/Tenacibaculum</i>
				<i>Mesonina</i>
				<i>Polaribacter</i>
				<i>Winogradskyella</i>
Deinococcus- Thermus	<i>Deinococci</i>	<i>Deinococcales</i>	<i>Deinococcalaceae</i>	<i>Dienococcus</i>
Proteobacteria	<i>Alphaproteobacteria</i>	<i>Rhodobacteriales</i>	<i>Rhodobacteriaceae</i>	<i>Ahrensia</i>
				<i>Phaeobacter</i>
				<i>Roseovarius</i>
				<i>Ruegeria</i>
				<i>Sulfitobacter</i>
		<i>Sphingomonadales</i>	<i>Sphingomonadaceae</i>	<i>Sphingomonas</i>
		<i>Rhodospirillales</i>	<i>Acetobacteraceae</i>	<i>Roseomonas</i>

Table 4.3. Continued

Phylum	Class	Order	Family	Genus
			<i>Comamonadaceae</i>	<i>Acidovorax</i>
	<i>Gammaproteobacteria</i>	<i>Altermonadales</i>	<i>Altermonadaceae</i>	<i>Achromobacter</i>
				<i>Alteromonas</i>
			<i>Colwelliaceae</i>	<i>Colwellia</i>
			<i>Shewanellaceae</i>	<i>Shewanella</i>
		<i>Pasteurellales</i>	<i>Pasteurellaceae</i>	<i>Pasteurella</i>
		<i>Pseudomonadales</i>		<i>Pseudoaltermonas</i>
				<i>Pseudomonas</i>
		<i>Vibrionales</i>	<i>Vibrionaceae</i>	<i>Vibrio</i>
				<i>Uncultured proteobacterium</i>

Microbial community figures 4.19, 4.20, and 4.21 demonstrate the frequency of identification of the top 36 genera at SNE (System 3), SME (System 2) and NME (System 1) respectively and figures present the 10 most reported genera associated with ESD; *Aquimarina*, *Chryseobacterium*, *Empedobacter*, *Flavobacterium*, *Polaribacter*, *Pseudoaltermonas*, *Roseomonas*, *Roseovarius*, *Sulfitobacter* and *Vibrio*. Each figure represents the 3 sampling time points; Baseline, Time 1 and Time 2. There is no distinct pattern of frequency occurrence over time and temperature for any system. Also the isolates most often associated with ESD were isolated from apparently health and shell diseased lobsters.

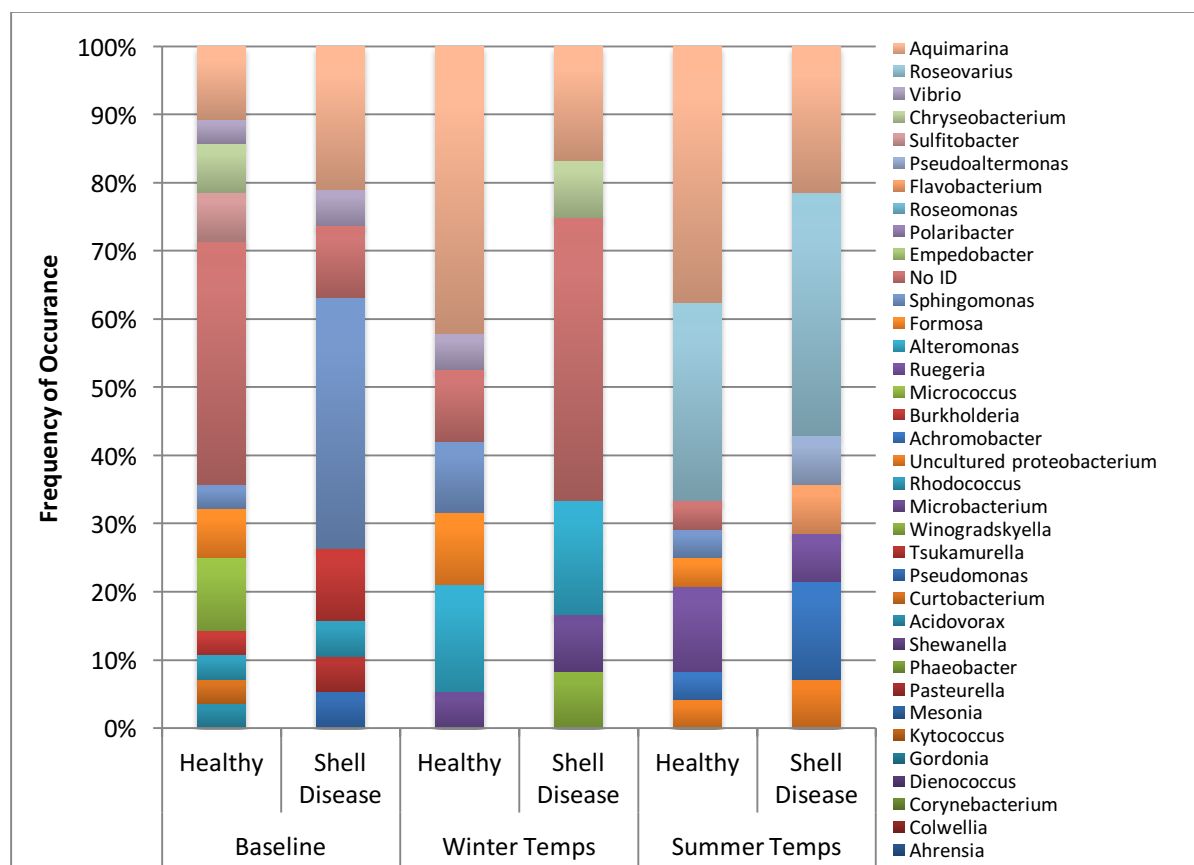


Figure 4.19. Frequency of isolation of bacterial genera for Southern New England (System 3, high temperature cycles) at each time point.

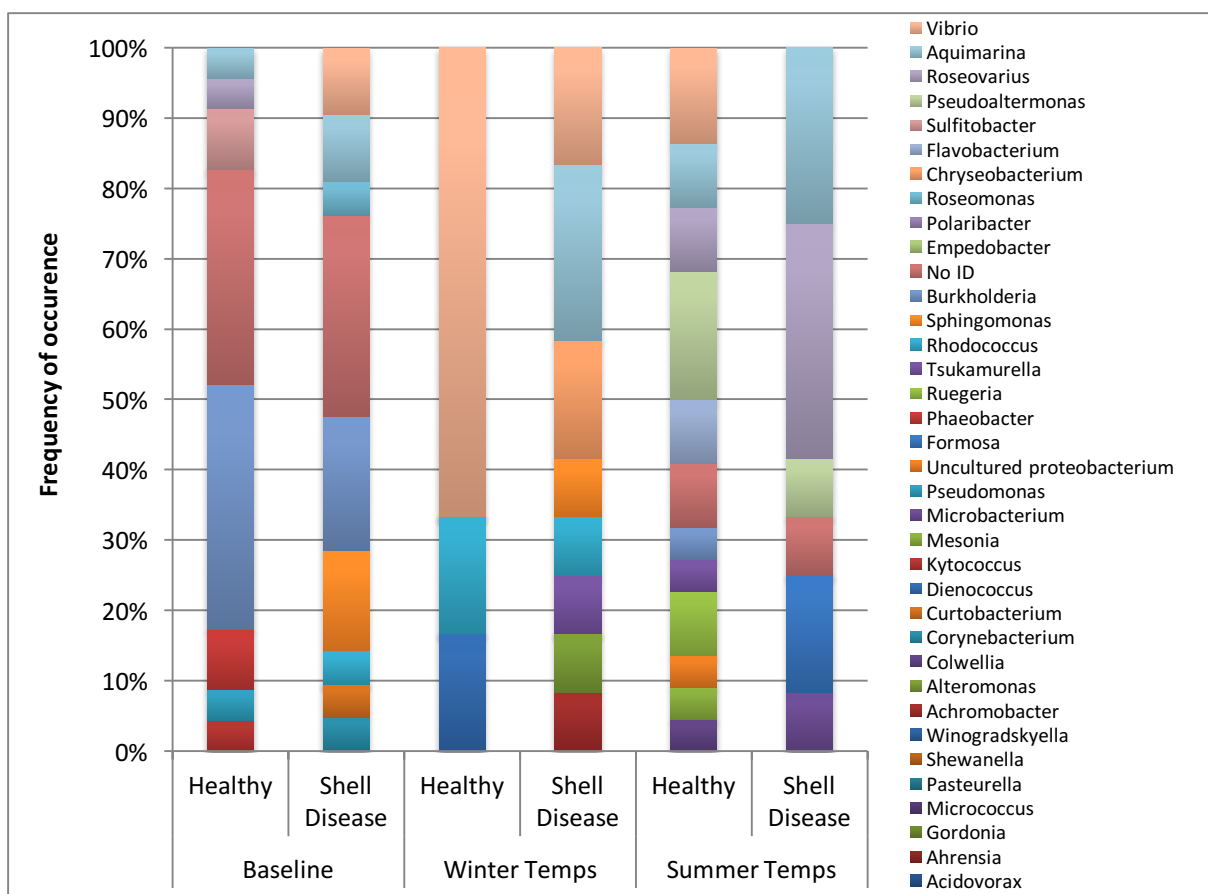


Figure 4.20. Frequency of isolation of bacterial genera for Southern Maine (System 2 mid-range temperatures) at each time point

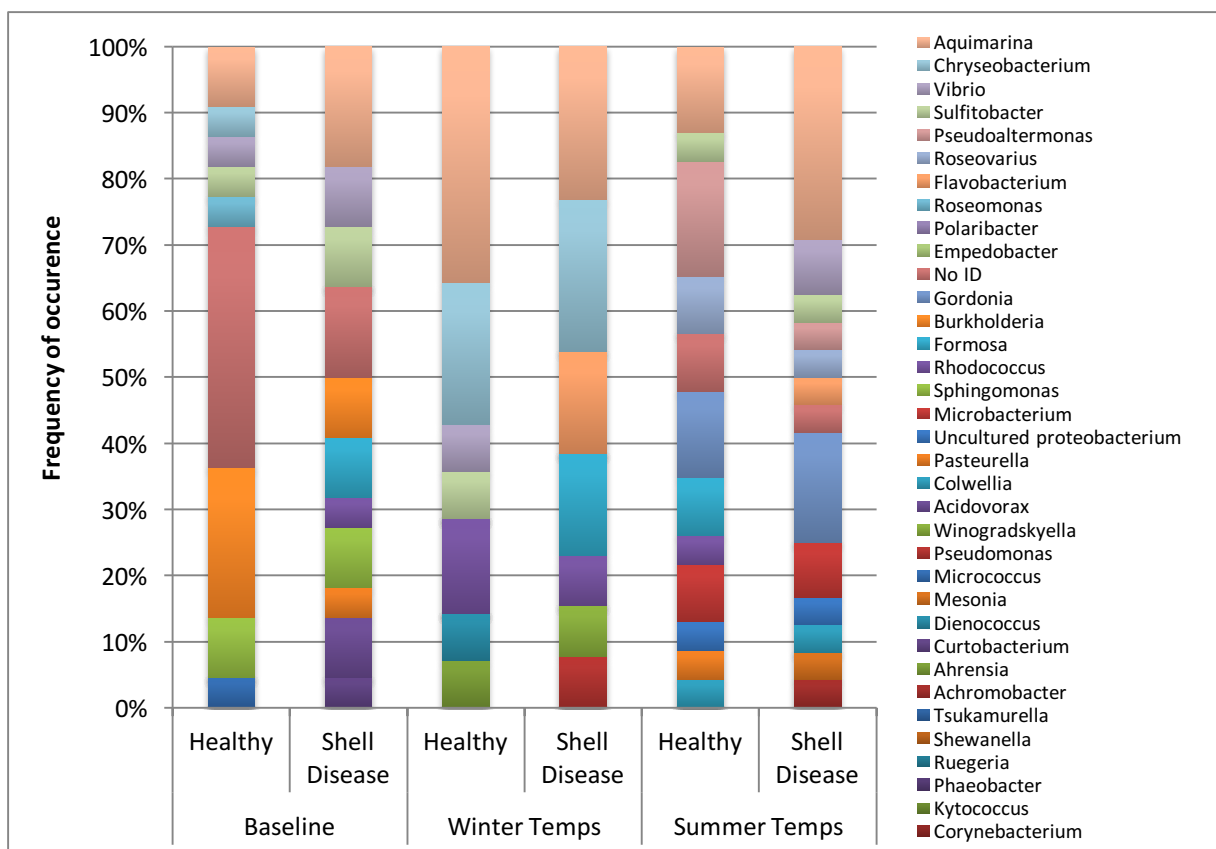


Figure 4.21. Frequency of isolation of bacterial genera for Northern Maine (System 1, low temperature cycles) at each time point

The Shannon-Weiner diversity index was calculated for each group at each time point (Table 4.4) and indicates that the diversity indices of all groups are relatively similar between healthy versus diseased and between sampling time points.

Table 4.4. Shannon-Weiner diversity indices calculated for each group.

	Shell Disease Status	Baseline	Time point 1	Time point 2
System 1: Low Temp	Non-Disease	2.12	1.86	2.37
	Disease	2.46	1.97	2.26
System 2: Mid Temp	Non-Disease	1.90	1.39	2.66
	Disease	2.16	2.10	1.81
System 3: High Temp	Non-Disease	2.32	1.90	1.65
	Disease	2.18	1.87	2.96

Discussion

Few longitudinal studies have been performed that follow disease progression of ESD in individual lobsters. Tlusty et al. (2008) used hatchery reared juvenile *H. americanus* to study the effects of diet on the development of shell disease but the study was not specific to ESD. Stevens (2009) examined the progression of ESD in a flow-through laboratory over a year in which high mortalities were claimed to be from complications of shell disease and also from high temperatures. Tlusty et al. (2012) also investigated the effects of fixed temperatures on shell disease in laboratory reared lobster populations and used diets as a stressor to help induce shell disease. Results from this study indicated that while temperature plays a role in the onset of shell disease, length of molt cycle was also a critical factor in shell disease progression. These described studies did not investigate the microbial community dynamics over time and temperature. Our longitudinal study examined the effects of increasing temperatures, reflecting seasonal temperature patterns of warming, rather than a single fixed temperature as it is more representative of natural conditions. We investigated the antimicrobial plasma activity as a measure of immune function in these lobsters. The enumeration and culture-dependent microbial community of lobsters were examined to evaluate changes in the microbial community profile and stability of apparently healthy and shell-diseased lobsters.

The seasonal temperature regimes achieved during this study were reflective of the average monthly temperatures obtained through NOAA's National Oceanographic Data Center (NODC, 2013)(Figure 4.1) for the three regional areas. The laboratory

recirculating systems were not able to reach the lowest indicated temperatures but systems were able to reach the highest reflected temperatures. Except for predetermined temperature changes, all water quality parameters remained constant throughout the study duration for each system.

During the course of our study, 61% of the healthy lobsters and 59% of the diseased lobsters molted. Similar to (Stevens, 2009), we observed minimal increase in observational ESD progression over the eleven months. The diseased lobsters that molted and re-developed signs of shell disease were in the mid and high range temperature groups. There was also a significant weight gain for all lobster groups with no significant difference between non-shell diseased and shell diseased lobsters over the course of the study. This was different than observed by Stevens (2009) who suggested slower growth rates due to ESD.

Low mortality was observed in healthy lobsters in all temperature regimes. While there were more mortalities in the shell diseased lobster than the healthy lobsters it was not significantly different. In the SME temperature regime, there was a trend for the shell diseased lobsters to have higher mortality than shell diseased. If our study had higher numbers of animals per treatment group this trend might have been better defined.

We examined the antimicrobial activity of the plasma based on work from Noga et al. 1994 and Homerding et al. 2012. Noga et al. used a turbidimetric assay effectively to determine the antibacterial activity of plasma from blue crabs, *Callinectes sapidus* with and without shell disease collected from natural populations from various

geographical sites. Reported results indicated site specific differences in serum antibacterial activity and also reported significant lower serum antibacterial activity in crabs with shell disease than crab without shell disease. Homerding et al. 2012 used this assay to determine the antibacterial activity of plasma from lobsters with and without ESD from two geographical locations. Results from this study did not measure significant differences in plasma activity between healthy versus shell diseased lobsters but a significant difference was observed based on geographical location. Our study results did not demonstrate significant bacterial growth inhibition; thus, no measurable antibacterial activity was seen in any of the lobster hemolymph over any of the time points when evaluating a particular group. The optical densities of individuals at testing time points had high inter-individual variability but the overall population results indicated no antibacterial response. The lack of antimicrobial activity in our study may indicate that in a controlled laboratory environment the stress factor of temperature alone did not result in a measurable plasma antibacterial immune response. In regards to the study by Homerding et al. 2012, there may have been other environmental stressors contributing to plasma response. Of note,(Noga et al., 1994) used serum (coagulated hemolymph with clot removed) and our procedure followed Homerding et al.(2012) and used plasma (centrifuged hemolymph prior to clotting to remove hemocytes). The innate immune response of decapod crustaceans has integrated cellular and humoral components, both cellular and plasma responses work in coordination (Haunton 2012). The procedure of immediately separating the hemocytes from the plasma may have reduced or eliminated antimicrobial proteins secreted from

hemocytes during the clotting process. Investigating the serum antimicrobial response may have provided measurable responses.

Microbial community sampling for each lobster was performed 3 times approximately 3 months apart reflecting seasonal temperatures of summer, winter and peak summer for each regional area described. The objective was to monitor the changes over time of microbial numbers and frequency of the most predominant organisms. The same anatomical location was swabbed on each lobster. For clarity, swabbing was not targeted specifically at diseased areas on lobsters with signs consistent with ESD but rather the same anatomical site on the lobster, no-shell diseased or diseased. Culture-dependent enumeration and identification of organisms were performed.

Baseline bacterial enumeration indicated that the bacterial loads on shell diseased lobsters were significantly higher than healthy lobsters. As seasonal temperature cycles progressed, the Time 1 sampling bacterial loads were significantly higher in the high (SNE) temperature range on both healthy and diseased over the mid (SME) and low (SNE) temperature range but in the high (SNE) and (mid) SME range there was no difference in bacterial load on healthy versus diseased. In the low (NME) temperature system significant higher bacterial loads were still observed on the diseased versus healthy lobsters. The final sampling, Time 2, was performed at peak summer temperatures for all systems. For this sampling, the bacterial loads were significantly higher in the high (SNE) and (mid) SME range over the low (NME) but again there was no difference observed in the bacterial load between healthy and diseased in

the high (SNE) and (mid) SME range. In the low (SNE) temperature range there was a trend for the bacterial load to be higher on the shell diseased. These results do indicate that temperature has an effect on the bacterial loads of lobster shells. Higher temperatures support higher bacterial loads. It is interesting that as seasonal temperature cycles progressed, results demonstrated to no significant difference in bacterial loads between diseased and healthy lobsters. It is assumed that the natural colonization of the microbial flora increased with increasing temperatures on the healthy lobster shells and maintained those bacterial loads in our aquaria systems for healthy and diseased lobsters.

When evaluating the microbial community profiles of isolated and identified bacteria, our focus was to compare the frequency of identified organisms associated with ESD and compare the frequency of isolation over time and temperature on apparently healthy and diseased lobsters. Using a combination of BMIS phenotypic identification and 16sRNA Sanger sequencing we did isolate and identify organisms from the *Aquimarina*, *Chryseobacterium*, *Empedobacter*, *Flavobacterium*, *Polaribacter*, *Pseudoaltermonas*, *Roseomonas*, *Roseovarius*, *Sulfitobacter* and *Vibrio* genera. These genera have all been reported as being associated with ESD (Chistoserdov et al. 2005, Bell et al. 2012, Chistoserdov et al. 2012, Meres et al. 2012). Considering *Aquimarina* and *Thalassabious* in particular, we did routinely isolate *Aquimarina* but we did not, however, identify *Thalassobious*. We did, however, identify closely related genera from the *Rhodobacteriaceae* family. Based on frequency of isolation, there was no distinct patterns of frequency of isolation and the isolates most associated with ESD were

isolated from both healthy and diseased lobsters present on all groups at all sampling time points. We calculated the Shannon-Weiner diversity index for all groups in order to determine the species diversity of all groups and time points. The diversity indices were similar for all groups. Our study results are similar to a study performed by (Whitten et al., 2014) that examined the bacterial species in the cuticles of European lobsters (*Homarus gammarus*) and American lobsters sharing the same aquarium systems.

By selecting the top five most predominant organisms observed by culture, we were able to determine frequency of isolation but not the quantity of each species isolated. However, our objective was to monitor the stability of the lobster shell microbial biofilm through seasonal temperature profiles reflecting SNE, SME and NME and to determine the effects of increasing temperatures on ESD. Overall, our results did indicate that temperature has an effect on the bacterial load on shells but over time the differences in bacterial load on diseased versus healthy shells was not significant in our systems. We did isolate and identify the organisms most often associated with shell disease on both healthy and diseased shells and the diversity of species was similar in all groups. In our systems, the higher temperature regimes did not exacerbate ESD progression and it did not detectably alter microbial communities. Based on our tank-based study, temperature alone does not affect the progression of ESD and indicates the complexity of disease occurrence and progression in diverse natural marine environments.

For future research, the comparison of the variability and complexity of the microbial community inhabiting the lobster carapace might be better characterized

using high throughput sequencing, which provides greater depth and discrimination among populations. High throughput sequencing generates thousands of sequences, which can be used to identify community members to the genus level. The rich dataset generated provides a more complete community profile and a better ability to identify differences between populations than could be obtained using older culture-free methods such as denaturing gradient gel electrophoresis (DGGE), which are limited to dozens of community members. It remains important to simultaneously compare culture-dependent enumeration and microbial community identification for comparison to previous research and to attempt to obtain cultures for use in experimental work in the laboratory for improvement of challenge models.

CHAPTER 5

SUMMARY AND FUTURE DIRECTIONS

Investigating the impacts of environmental stressors on host-pathogen interactions remains critical. Environmental changes, such as increasing temperatures, have been linked to new and emerging infectious diseases in natural populations of *Homerus americanus*. These studies provided further insight into the etiologies of limp lobster disease (LLD) and epizootic shell disease (ESD), two diseases of that continue to cause significant mortality in natural lobster populations.

Mortality from LLD occurs in natural populations but is more intense in impounded lobsters. *Photobacterium indicum* is the bacterial agent associated with LLD. Results from our study more clearly define the community ecology of this suspected opportunistic bacterial pathogen. *P. indicum* was found to be a common member of the microbial communities of the branchial chamber and intestinal mucosa of freshly captured and impounded *H. americanus*, and was also isolated from the lobster pound sediment. These results support the opportunistic nature of this organism as a pathogen.

Despite the widespread use of an FDA approved oxytetracycline dehydrate medicated feed, *P. indicum* has been consistently isolated from moribund and dead lobsters from pound surveys. This medicated feed is not approved for use in controlling *P. indicum* and other gram negative bacteria. For this reason, the use of oxytetracycline dehydrate medicated feed is contraindicated. As an alternative to antibiotic treatment,

a *P. indicum* bacterin was produced and used as an immunostimulant to stimulate a short-term innate protective immune response in adult American lobsters. Our study did not demonstrate a significant change in measurable immune response through examining total circulating hemocytes and the respiratory burst activity of hemocytes. However, when used as an immersion bath, the prepared bacterin was safe to use with lobsters and no adverse reactions were observed. Future studies to compare dose administration of the bacterin as an immersion and injection is warranted. A bacterial challenge with *P. indicum* could more clearly determine the efficacy of the bacterin to produce short-term protection in lobsters post stimulation.

Epizootic shell disease is an aggressive form of shell disease described as having a multifactorial and complex etiology likely involving multiple microbes. The microbial genera consistently associated with ESD are *Aquamarina* and *Thalassobious* species but a true etiology has yet to be defined. Our laboratory-based longitudinal study examined the effects of three seasonal temperature cycles on the immune response and progression of ESD in adult female lobsters. Particular attention was placed on the stability of the lobster shell's microbial community stability over time and temperature. Observationally, there was very little increase in ESD progression throughout the study. Low mortality was observed in the non-shell diseased lobsters but there was a trend for higher mortality in the diseased-lobsters in the mid-range and high temperature seasonal cycles. No measurable immune response was observed in any of the lobsters throughout the study using the lobster antimicrobial assay. Bacterial enumeration data suggest that temperature and shell disease influence bacterial loads. By culture

dependent techniques, we isolated and identified the organisms most often associated with ESD. We did routinely isolate *Aquimarina* but we did not identify *Thalassobious*. The organisms most often associated with shell disease were detected on diseased lobsters as well as lobsters with no apparent shell disease. Over all, the culture dependent microbial diversity remained relatively stable over time and temperature suggesting that increasing temperatures alone did not exacerbate ESD progression or alter the microbial communities.

For future research, the comparison of the variability and complexity of the microbial community inhabiting the lobster carapace utilizing Next Generation Sequencing techniques could be used to identify community members to the genus level. The rich dataset generated would provide for a more complete community profile and a better ability to identify differences between populations. It also would determine the relative abundance of microbial taxa. It remains important to compare culture-dependent enumeration and microbial community identification with Next Generation Sequencing results to better define culture-dependent versus culture independent microbial profiles. It may be prudent to further investigate immune response of lobsters using the serum for the antimicrobial response as a measure of immune function. Taken together this work could be used to possibly design more robust challenges for further examining disease etiology.

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Deborah Anita Bouchard is an aquatic animal health professional with interests in applied aquatic animal research and diagnostics. Deborah graduated from the University of Maine in 1983 with a Bachelor of Science degree in Microbiology. She worked as a professional at the University in the Microbiology department in research and teaching until 1990. She left the University to work in the private sector where she gained experience in human clinical microbiology, aquatic animal diagnostic and inspection work, food microbiology, and whole effluent toxicity testing. In 1996, Deborah co-founded and served as the President and CEO of Micro Technologies Inc, a biotechnology company providing services to the US and Canadian aquaculture industries. In 2004, she sold her share in the company, did a short stint at the Maine Department of Marine Resources in aquatic animal health policy and in 2006 returned to the University of Maine, as the laboratory manager of the University of Maine Cooperative Extension's newly created Maine Aquatic Animal Health Laboratory. Deborah's position has transitioned to a split position with the University of Maine Cooperative Extension and the Aquaculture Research Institute as the Laboratory Manager and Aquatic Animal Health Industry Research Coordinator. In the summer of 2017, she was appointed as the Director of the Aquaculture Research Institute. Deborah is a candidate for the Doctor of Philosophy Degree in Aquaculture and Aquatic Resources from the University of Maine in August 2018.